ROLES OF BITING PREFERENCE AND INSECTICIDE RESISTANCE IN DETERMINING MALARIA TRANSMISSION HETEROGENEITY IN THE GAMBIA

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DECLARATION

This work has not previously been accepted in substance for any degree and is not being currently submitted in candidature for any degree

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This thesis is the result of my own investigation, except where otherwise stated. Other sources are acknowledged and bibliography is appended

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DEDICATION

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To my late parents Alloys Opondo and Emily Akala, thank you for bringing us up, we have made your names soar the skies in our times, may you Rest in Eternal Peace

To Ludwig and the future of Humanity and Mankind, may this work contribute to making Earth a better place to live.

ABSTRACT

Malaria continues to cause immense public health pressure to poor and developing countries in the tropical world hindering economic development. Malaria control using long-lasting insecticide-treated bed nets (LLINS) and indoor residual spraying (IRS) have contributed significantly to the reduction of malaria transmission. However, this reduction in transmission is not uniform. Particular foci 'hotspots' that do not respond as anticipated to control tools have become common in low-moderate transmission settings like The Gambia. While several factors contribute to heterogeneity in malaria transmission, the roles that malaria vectors with differing behaviour, ecology and susceptibility to insecticides play in these hotspots is poorly understood. This thesis aimed to investigate whether vector-related factors might contribute to malaria transmission heterogeneity in The Gambia.

First, I demonstrated how the three major malaria vectors; *Anopheles gambiae* s.s., *Anopheles arabiensis* and *An. coluzzii* varied in relative abundance between villages of varying transmission intensities. Susceptibility to deltamethrin and DDT varied significantly among species and locales with *An. gambiae* s.s. from the east of the country by far the most resistant. There was a correlation between *An. gambiae* s.s. phenotypic resistance to DDT and malaria transmission in 2013, driven by the east of the country which experiences higher malaria transmission.

The major driver of insecticide resistance to DDT and deltamethrin was the target site mutation, *Vgsc*-1014F although another target site mutation *Vgsc*-1575Y and a metabolic gene variant *Gste2*-119T were also implicated, albeit to a far lesser extent. The frequency of *Vgsc*-1014F in *An. gambiae* s.s. remained low in a second sampling year in western populations but approached fixation in in the east. Generally, whether resistant or susceptible, malaria vectors in the study villages primarily fed on humans, except *An. funestus*, sampled for the first time in the centre of the country, which fed more on cows. As a preliminary study, I investigated whether resistant populations naturally exposed to insecticides in the wild would still be resistant when older, since laboratory studies typically suggest a decline with age. Results suggested that selection by insecticides may result in resistance remaining in older mosquito cohorts, and marker-based prediction of resistance remained effective.

Finally, I used microsatellites to study population genetic structure of *An. gambiae* s.s. to investigate the origin and likelihood of spread of the strong insecticide resistance phenotypes in the east of The Gambia. Results suggested that although distance was the primary isolating factor among population samples, different ecologies representing coastal marine and drier inland ecosystems may also play a part in partitioning populations from the east and west of the country, likely maintained by large scale rice farming in the central area, dominated by *An. coluzzii*. Strong differentiation of eastern populations from Senegalese samples did not support a hypothesis of gradual spread of resistance, and *Vgsc*-L1014F differentiation far exceeded the neutral signal implicating local selection. Resistance to pyrethroids and DDT observed in The Gambia either represented one or more *de novo* mutations or rare migrants from Senegal subjected to varying local selection pressures.

The results presented in this thesis highlight how local variation in characteristics of vector populations is important in driving malaria heterogeneity. To tailor control interventions to tackle high malaria transmission zones, it will be useful for National Malaria Control Programmes to investigate the type of malaria vectors present, their behaviours and insecticide resistance profiles.

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LIST OF ABBREVIATIONS

LLIN	Long Lasting Insecticide Treated bed Net
IRS	Indoor Residual Spraying
GNMCP	Gambia National Malaria Control Programme
CDC-LT	Centres for Disease Control miniature Light Trap
EIR	Entomologic Inoculation Rate
ITN	Insecticide Treated bed Nets
Kdr	*Knockdown resistance*
HLC	Human Landing Catch
ELISA	Enzyme Linked Imunosorbent Assay
PCR	Polymerase Chain Reaction
S.s.	Sensu stricto
S.l.	Sensu lato
SSA	sub-Saharan Africa
Pf	*Plasmodium falciparum*
Vgsc	Voltage-gated sodium channel gene
URR	Upper River Region
CRR	Central River Region
LRA-N	Lower River River Region-North bank

LRA-S	Lower River Region-South bank
LRA-W	Lower River Region-West
EA	Eastern Area
GMEC	Global Malaria Eradication Campaign
MRCG	Medical Research Council Unit The Gambia
LSTM	Liverpool School of Tropical Medicine
DCE	Disease Control and Elimination theme

1 CHAPTER ONE: INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Disease prevalence varies at both macro and micro spatial scale where individuals within communities experience more disease than others (Woolhouse et al., 1997). Consequently, due to heterogeneity in transmission, patterns of disease where majority of cases are aggregated or clumped in small geographically defined areas have been defined as hotspots of transmission (Bejon et al., 2010).

These hotspots are important because they may act as sources of transmission to new areas. Malaria transmission often shows this heterogeneity (De Beaudrap et al., 2011, Kreuels et al., 2008). The factors that maintain these hotspots are not well understood but with renewed efforts toward malaria eradication, understanding what drives these heterogeneities will be crucial.

The identification of these hotspots is a first step toward investigating and then understanding their drivers (Bousema et al., 2010). Although not easy to identify in high malaria endemic regions due to high prevalence of disease and prolonged presence of circulating antibodies (Smith et al., 1993), Kreuels *et al* in Ghana observed spatial variation in disease incidence in villages from an endemic area (Kreuels et al., 2008). Where malaria transmission is low, hotspots are expected to be more apparent (WHO, 2007).

Malaria control has relied upon Long Lasting Insecticidal bed Nets (LLINs) and Indoor Residual Spraying (IRS) to control indoor biting malaria vectors. These interventions have contributed significantly (about 60%) to the observed decline in malaria related deaths and morbidity (WHO, 2015b) and to the prevention of 663 million clinical cases (Bhatt et al., 2015). With further control and renewed commitments (WHO, 2015a), disease control

programmes will therefore be faced with these persistent hotspots of transmission and that will form the final hurdles for malaria elimination.

In The Gambia, where transmission is relatively low and continues to decline (Ceesay et al., 2010), prevalence of infection can vary substantially between neighbouring communities (Mwesigwa et al., 2015, Okebe et al., 2014). Also, the four main malaria vectors vary in space (Caputo, 2008) and time (Lindsay et al., 1993a), with different degrees of insecticide resistance (Betson et al., 2009, Tangena et al., 2013). Furthermore, recent data showed malaria vector population sub-structuring between west (coast) and east (inland) of the country with clear genetic differences that may enable them to colonize different ecological zones (Caputo et al., 2014).

As part of a larger study investigating the transmission dynamics of malaria in heterogeneous transmission settings of The Gambia, we identified villages of varying transmission intensities following a country wide malaria cross sectional survey in 2012 (Mwesigwa et al., 2015). A pair of villages was identified in each of the 6 study regions. In each pair, one village had high and the other low malaria prevalence. The study, spanning three years beginning 2013 aimed to determine environmental, human, vector and parasitological factors contributing to malaria heterogeneity in The Gambia.

This thesis addressed whether different malaria vectors with varying blood feeding behaviours and insecticide resistance profiles contribute to the observed heterogeneity of malaria burden in The Gambia.

1.1.1 Goal

Determine whether variation in species composition, their insecticide resistance profiles and blood feeding choices contribute to observed heterogeneity in malaria transmission in The Gambia.

1.1.1.1 Specific objectives

a) Characterize the species and molecular form composition of the mosquito fauna in varying malaria transmission settings in The Gambia

b) Characterize the patterns of phenotypic resistance and known molecular markers of resistance allele frequency (*Vgsc*-1014F, *Vgsc*-1575Y, *Ace-1*-119S).

c) Determine the relationship between age and insecticide susceptibility of wild caught anophelines to DDT and deltamethrin

d) Determine host preference behaviours of malaria vectors via molecular blood meal analysis

e) Determine macro and micro-spatial distribution of species, their sizes and connectivity of populations

1.2 Literature Review

1.2.1 Malaria transmission

Malaria is a parasitic disease that is caused by five parasites of the genus *Plasmodium* namely; *P. falciparum, P. malariae, P. ovale, P. vivax* and *P. knowlesi* (Guerrant et al., 2004). Of the five *Plasmodium* species, *P. falciparum* (*Pf*) has long been known to be most dangerous, causing highest morbidity and mortality in humans (Bass, 1920) (WHO, 2015b), and the dominant malaria species across sub-Saharan Africa (SSA) (**Figure 1**). It is transmitted from human to human via the bite of a female mosquito vector of the genus *Anopheles*.

In Africa, the most important afro-tropical malaria vectors belong to the *Anopheles gambiae* and *Anopheles funestus* complexes (Gillies MT and De Meillon BT, 1968, Gillies and De Mellion, 1968). *An. gambiae* sensu lato comprises of *An.gambiae* sensu stricto, where formerly in West Africa it was further divided into molecular forms, S and M (now called *An. coluzzii*), *An. arabiensis*, *An. melas* (West Africa) and *An. merus* (East Africa).

The quantification of malaria transmission and epidemiology has incorporated various aspects of the vector, human and parasite. Malaria transmission intensity, upon which the success of its control is measured, can be measured by several indices such as the entomologic inoculation rate (EIR), prevalence of infection in the human population and the vectorial capacity (C) (Garrett-Jones and Shidrawi, 1969).

The EIR and prevalence are commonly used to estimate malaria burden. The EIR, defined as the number of infectious bites an individual receives in a specified period of time is expressed as product of vector biting rate times the proportion of mosquitoes infected with sporozoite-stage malaria parasites (Beier et al., 1999). Prevalence is expressed as the proportion of

persons either infected with the parasites or having antibodies against the parasite in a population (WHO, 2015b).

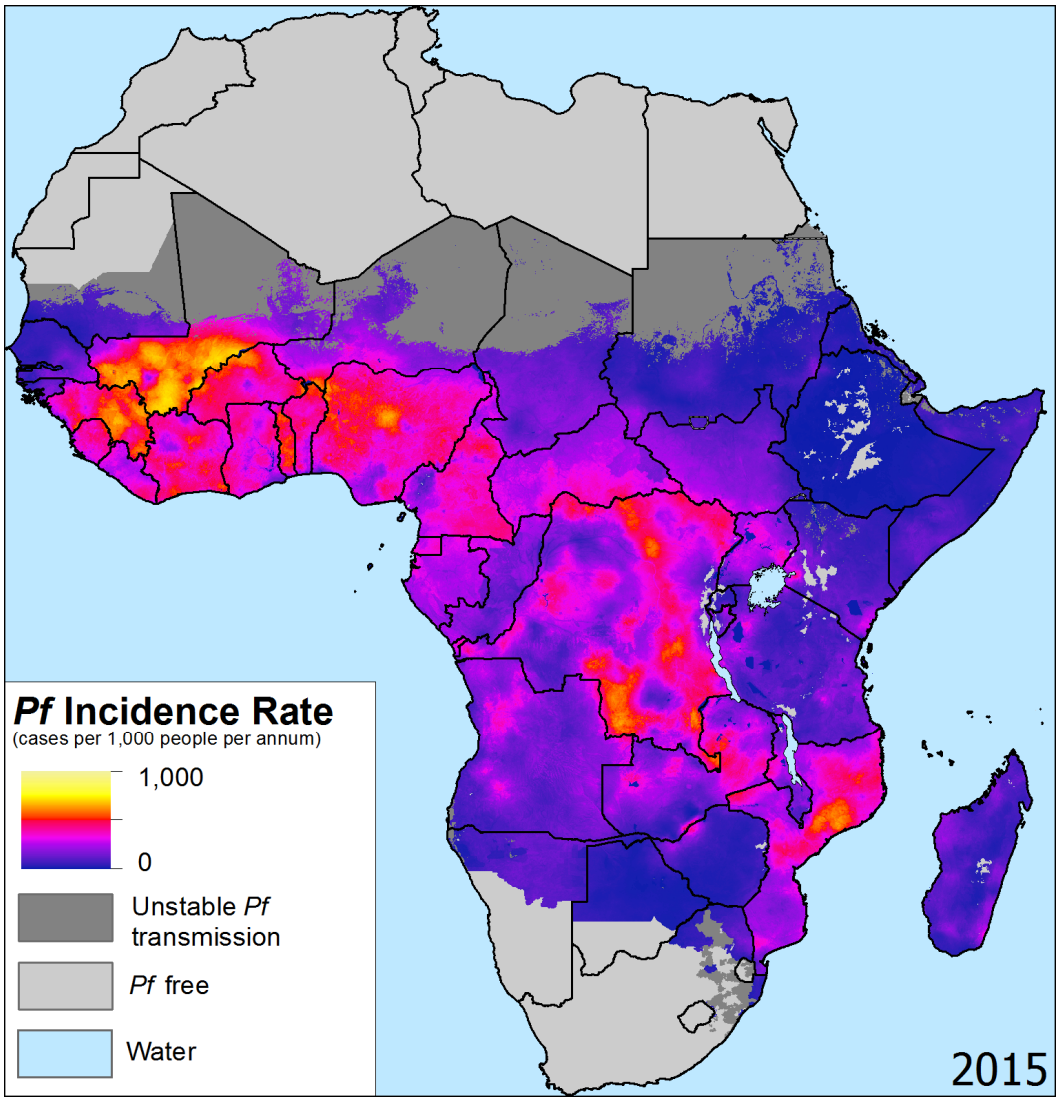

Figure 1: Distribution of malaria incidence rates in sub-Saharan Africa. Image source: Malaria Atlas Project, http://www.map.ox.ac.uk/

1.2.2 Epidemiology of malaria

Malaria in Africa has caused enormous economic burden on Africa's inhabitants with an estimated annual loss of USD $12 billion as a result of reduced human productivity and redirection of resources toward health care and malaria control (Breman et al., 2004, Gallup and Sachs, 2001). Malaria in Africa continues to contribute over 90% of malaria cases in the world (WHO 2015).

Commonly malaria transmission in SSA shows seasonal periodicity with low transmission during dry seasons with peaks of transmission and disease usually seen during or just after the rainy/wet seasons (Greenwood et al., 1987, Ceesay et al., 2010, Hawley et al., 2003). Regardless of this seasonality, transmission is not uniform across all settings thereby creating differences in transmission intensities resulting in high and low malaria transmission zones even on a small geographical scale (Mbogo et al., 2003, Woolhouse et al., 1997).

Where transmission is intense, incidence rates of malaria infection and clinical diseases are extremely high, and transmission is usually perennial and less affected by seasonal fluctuations in weather patterns (Smith et al., 1993). These areas have been described as stable malaria transmission zones and are characterized by high transmission rates, higher vector density to human ratio, higher exposure to infective mosquito bites per person per year and due to the acquisition of partial immunity with age (Hviid, 2005), the most high risk group are children below the age of five and pregnant mothers.

Malaria transmission is defined as unstable when transmission intensity varies by year as is often the case for urban areas, highlands and arid areas (Mala et al., 2011, Hay et al., 2005, Paaijmans et al., 2009). Malaria risk does not vary by age as population immunity is low because of low exposure to infection. In addition, access to basic preventive measures and health care services may also contribute to low malaria risk in urban settings.

The factors that have been suggested for explaining the heterogeneity of malaria transmission, though not mutually exclusive, can generally be grouped as resulting from human behaviour, vector behaviour and environmental factors.

1.2.3 Malaria heterogeneity and its possible causes

The drivers of heterogeneity in malaria transmission can be thought of as any factor that either increases or reduces human exposure to infective mosquito bites. Humans that spend time outdoor unprotected at night (Huho et al., 2013) or indoor unprotected before going to sleep under a bed net (Seyoum et al., 2012, Thomson et al., 1994) have a higher risk of acquiring malaria if infective bites are diverted to them (Quiñones et al., 2000).

Additionally, humans whose frequent movements coincide with the vector biting times might have higher risk of malaria compared to those that either do not move or less exposed to bites (Stoddard et al., 2009). The roles that human behaviour may play in driving malaria heterogeneity can be drawn from studies of Asian malaria and malaria in the Amazon where *P. vivax* is the main malaria parasite (Price et al., 2007). In Peru for example farmers who visited forested areas for farming, logging of trees and mining were more likely to have malaria than those who did not. Furthermore, family members who stayed with them were likely to have malaria as well (Chuquiyauri et al., 2012).

In Philippines (Lansang et al., 1997) and Vietnam (Erhart et al., 2005), frequent visits to the forests at night increased the odds of men having malaria. Similar trends have been observed in other parts of SSA like Ethiopia where the risk of malaria increased for individuals who were migrating to malarious low lands to seek work (Ghebreyesus et al., 2000). In SSA

where domestic agriculture is practiced, early rise to go to farmlands might increase exposure to outdoor and early morning biting mosquitoes.

Man-made modification of environments like building of dams and irrigation farming creates breeding sites which may increase vector density. If these modifications occur in unstable or low malaria zones, communities that are contiguous to these features often experience higher malaria compared to areas further away (Khaemba et al., 1994, Ghebreyesus et al., 1999).

This exposure to higher vector density due to proximity to naturally occurring breeding habitats is also commonly observed (Minakawa et al., 2002, Lindsay et al., 1995, Zhou et al., 2007) and may lead to increased malaria risk in unstable malaria zones (Trape et al., 1992, Githeko et al., 2006). However, in a region of relatively higher malaria transmission this exposure may not result in high malaria prevalence (Diuk-Wasser et al., 2005, Dolo et al., 2004).

The relationship between vector density and malaria transmission is complex particularly in high transmission zones. Higher biting rates are observed in areas near breeding habitats while infectious bites undertaken by older mosquitoes occur further away from these breeding habitats (Smith et al., 2004).

Mathematically, as vector density increases, so does malaria prevalence and (EIR) until saturation point is achieved where the maximum number of infections has been achieved and hence any new additional bites would result in super infection (Macdonald, 1950). This rise in density and transmission intensity is best observed in low transmission settings such as coastal regions, highlands, arid areas and urban settings where density of malaria vectors is generally low but which rises with precipitation (Trape et al., 1992) (Lindblade et al., 1999).

Wind direction also contributes to malaria heterogeneity; malaria prevalence among individuals residing in houses located on the upwind direction relative to breeding habitats will have more malaria compared to those residing in houses located on the downwind (Midega et al., 2012). Host odour from dwellings are carried by wind towards the breeding habitats hence mosquitoes will tend to move upward the wind direction (Lindsay et al., 1995) but downwind movement has also been observed (Gillies, 1974).

During their search for a blood meal mosquitoes may fly from few metres to several hundreds of metres up to kilometres depending on the availability of vertebrate hosts (Service, 1997). The time that these vectors seek human hosts, whether indoor or outdoor may bring about marked differences in transmission.

Although the level of anthropophagy, endophily and exophily differ between species (White and Rosen, 1973), vector control have affected host seeking patterns (Lindsay et al., 1993a). Thus, in places where the vectors feed exclusively on man, more transmission may occur than in places that the mosquitoes have alternative hosts to feed on (Killeen et al., 2001, Koella, 1991).

Today, vector control tools have either eliminated species that predominantly fed indoor leaving proportionately high outdoor biters (Bayoh et al., 2010, Russell et al., 2010) or have resulted in indoor biters altering their feeding and resting behaviour (Mbogo et al., 1996, Reddy et al., 2011).

Houses which either allows mosquitoes in or not through the eaves or windows also contributes to heterogeneity (Lindsay et al., 2003, Oesterholt et al., 2006, Ogoma et al., 2010). In rural areas where houses permit mosquito entry compared to those in urban settings, more malaria is observed (Kamya et al., 2015). However, socio-economic factors that affect

the purchasing power or access to basic preventive measures also contribute to this observed difference (Gardiner et al., 1984). Even in urban settings socio-economic differences among urban denizens leads to differential malaria transmission (De Beaudrap et al., 2011, Keiser et al., 2004).

Urban areas present special scenarios that contribute to the overall malaria heterogeneity observed in SSA and other parts of the world (Robert et al., 2003) and will be important in the last push to eliminate and eradicate malaria (Donnelly et al., 2005). Africa is rapidly urbanizing with 40% currently living in urban areas and is projected to reach 56% in 2050 (UN, 2014).

When urbanization is not planned, disease risk might be manifold especially among the poor (Phillips, 1993, Hay et al., 2005). Indeed urban agriculture commonly practiced by the less rich dwellers has been linked to higher malaria risk (Klinkenberg et al., 2008, Klinkenberg et al., 2006, Klinkenberg et al., 2005). In such agricultural settings, creation of artificial breeding habitats due to irrigation and water use allows mosquito vectors to grow and increase chances of disease transmission (Dongus et al., 2009).

Social activities especially in towns where people spend a lot of time outside could result in heterogeneities and/or promote change of mosquito host seeking behaviours that impact malaria transmission and hence control (Lefevre et al., 2010, El Sayed et al., 2000, Stoddard et al., 2009). While urbanization generally clears natural breeding habitats, malaria vectors can adapt to new breeding habitats that are polluted (Awolola et al., 2007, Sattler et al., 2005) and depending on their distribution in space, malaria transmission is expected to vary.

1.3 Malaria control and insecticide resistance

1.3.1 Insecticides used in malaria vector control

Insecticides or pesticides are toxic chemicals that are commonly used to control pests and insects of public health and agricultural importance. These insecticides are grouped into various classes based on their mode of action on the insects (**Table 1**). Four main classes are approved for use in public health. For malaria control, pyrethroids impregnated in LLINS (**Figure 2**) and DDT in IRS have been the most commonly used because they are cheaper and less toxic.

Table 1: Classes of insecticides used in public health and their target sites of action

Group	Sub-group	class	insecticides	Primary target site of Action
1	A	Carbamates	Propoxur, bendiocarb, carbosulfan	Acetylcholinesterase (AchE) inhibitors
	B	Organophosphates	Malathion, fenitrothion, Pirimiphos methyl	
3	A	Pyrethroids & pyrethrins	Permethrin, deltamethrin, lambda-cyhalothrin, cyfluthrin, etofenprox, transfluthirn	Sodium channel modulators
	B	Organochlorines	DDT, dieldrin	

Adapted from Insecticide Resistance Action Committee (IRAC) (IRAC, 2010)

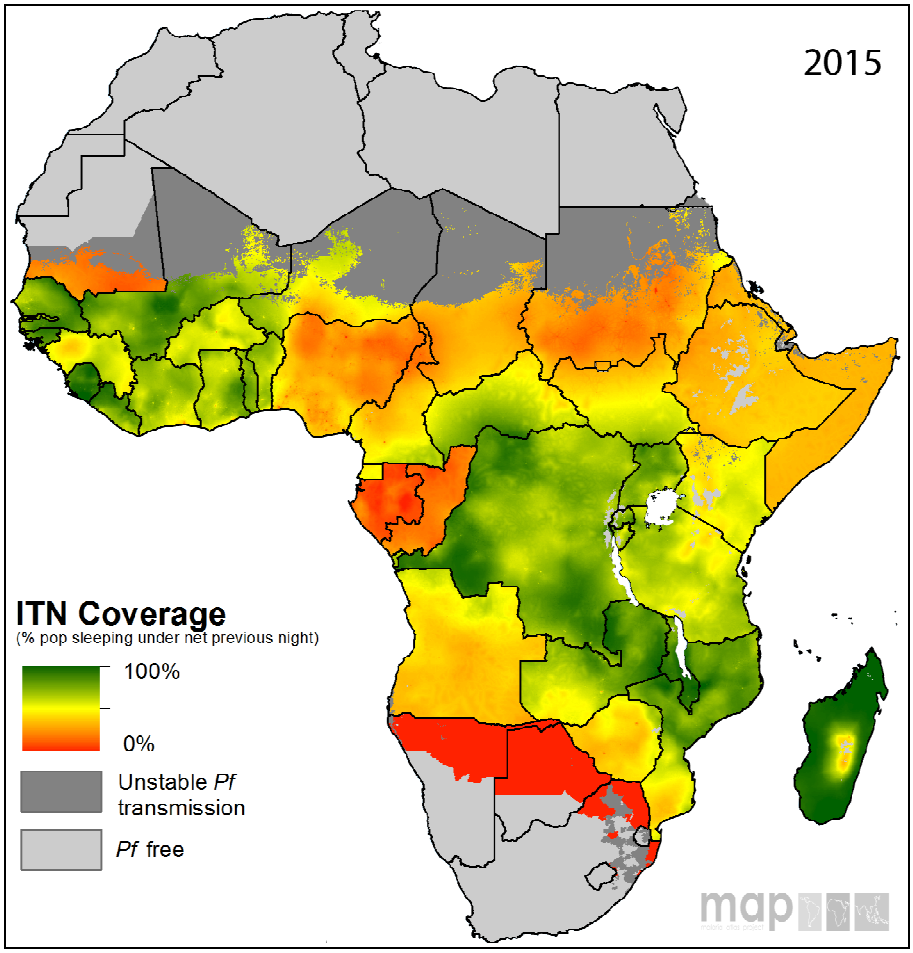

Figure 2: Coverage of Insecticide Treated bed Nets (ITNs) or Long Lasting insecticide Treated bed Nets (LLINs) for malaria control in Africa. Image source: Malaria Atlas project, http://www.map.ox.ac.uk/

1.4 Insecticide resistance and its mechanisms

According to WHO, insecticide resistance refers to the ability of insects like mosquitoes to withstand the effects of insecticides exposed to them by becoming resistant to its toxic effects by means of natural selection or mutations. It is a heritable change in the sensitivity of a pest population that is reflected in the repeated failure of a product to achieve the expected level of control when used according to the label recommendations for that pest species (Insecticide Resistance Action committee). Following use of insecticides for public health

and in agriculture, resistance against those insecticides have emerged (**Figure 3**) (Antonio-Nkondjio et al., 2011, Diabate et al., 2002, Ranson and Lissenden, 2016).

Different malaria vectors will have varying levels of resistance to insecticides based on either their inherent traits (endophily/endophagy) (Padonou et al., 2011, Kloke et al., 2011) or interaction with the insecticides in time and space (de Zulueta, 1959). Hence, because of this difference in exposure to insecticides, resistance development and therefore impact on malaria is not uniform (Molineaux et al., 1979).

For example, the more zoophilic mosquitoes like *An. arabiensis,* which may have minimal contact with the insecticides used peri-domestically are less likely to develop resistance to the particular active ingredient in the insecticide compared to those with more anthropophilic traits (Stump et al., 2004, Hargreaves et al., 2000). Alternatively, those not resistant could be representing populations that are less diverse and therefore less likely to evolve into resistant populations.

Although many resistance mechanisms have been described, only two; target site and metabolic have been extensively studied in malaria vectors. A third mechanism involving behavioural adaptation in response to vector control has recently been recognized (Gatton et al., 2013) but together with cuticular resistance, they have been studied less in malaria vectors.

1.4.1.1 Target site mutations

Many insecticides target neurone membrane ion channels and aim to interfere with their normal neurone functions (Narahashi, 2002). Target site mutations conferring resistance to DDT/pyrethroids involve amino acid substitution in the voltage-gated sodium channel (*Vgsc*) gene that alter the binding site of DDT and pyrethroids in the insect neurones.

This makes the chemicals not bind in their target site rendering them ineffective (Lund and Narahashi, 1983). Two types of substitutions at the 1014 codon of the *Vgsc* gene that replaces leucine with phenylalanine (Martinez-Torres et al., 1998) or serine (Ranson et al., 2000) have been unequivocally linked to DDT and pyrethroid resistance pyrethroid resistance (Ranson et al., 2004, Reimer et al., 2008, Sharp et al., 2007). Another mutation at codon 1575 that replaces asparagine with tyrosine was recently shown to confer additional resistance to DDT (Jones et al., 2012a).

Other target site mutations to the previously less commonly used insecticides bendiocarb and dieldrin have also been observed (Du et al., 2005, Weill et al., 2004). Mutation at the *Ace-1* gene, encoding Acetylcholinesterase (AChE enzyme), and result in replacement of glycine with serine at codon 119 makes the enzyme insensitive to bendiocarb and organophosphates (Weill et al., 2002, Djogbenou et al., 2007). Substitution of alanine with either serine or glycine at codon 296 of the GABA receptor gene (*Rdl*) makes the GABA receptor insensitive to dieldrin (Kwiatkowska et al., 2013).

1.4.1.2 Metabolic resistance

Metabolic resistance commonly involves insects increasing the production of enzymes involved in detoxifying the insecticides (Müller et al., 2008, David et al., 2005). These enzymes involved in detoxification belong to 3 main classes; Cytochrome P450 monooxygenases, Glutathione-S-Transferases (GST) and Carboxylesterases (Bergé et al., 1998, David et al., 2005, Djouaka et al., 2008, Mitchell et al., 2012, Wondji et al., 2009).

Metabolic resistance is the major mechanism of resistance in *An. funestus* complex but recently, a mutation in the *Ace-1* gene was associated with resistance to bendiocarb in South Africa (Ibrahim et al., 2016). Crucially, insecticide resistance mechanisms are not mutually exclusive. Target site mutations or metabolic mutations alone never account for full

variability in insecticide resistance profiles (Hemingway et al., 2013) and can combine to confer stronger resistance profiles (Mitchell et al., 2014) among resistant individuals.

1.4.1.3 Behavioural resistance

This arises mainly during host seeking and resting behaviours where vectors avoid contact with surfaces treated with insecticides or exit early from houses with insecticides (Quiñones et al., 1998, Mathenge et al., 2001, Ogoma et al., 2014). This perhaps could be due to the excito-repellency effect that insecticides have on these vectors (Chareonviriyaphap et al., 2004). When insecticides are intensely used indoor, vectors that feed outdoor might be selected for (Taylor, 1975) but whether this outdoor feeding is a heritable trait and caused by single or multiple changes in genes is less established.

Behavioural resistance has best been demonstrated in agricultural pests where pests avoided contact with surfaces that had insecticides on them (Sparks et al., 1989). Malaria vectors have also altered biting patterns where they feed on humans outdoor early in the evening before humans retire to use the bed nets during sleep (Reddy et al., 2011, Russell et al., 2011, Yohannes and Boelee, 2012) or early morning just as people wake up (Moiroux et al., 2012). Extreme case was observed in Senegal where *An. funestus* were reported biting in broad day light as a response to vector control (Sougoufara et al., 2014).

In addition, inaccessibility to humans has led to vectors being diverted to feed on other hosts in an attempt to avoid coming into contact with insecticides (Ndiath, 2014). This diversion however is beneficial if it reduces malaria transmission (Killeen et al., 2001).

1.4.1.4 Cuticular resistance

Cuticular resistance involves those changes in the cuticle which reduce their permeability to insecticides hence reduced insecticide uptake (Ahmad et al., 2006). Up-regulation of three

genes, CPLCG3, CPLCG4 and CPR21 encoding cuticular proteins may contribute to cuticular resistance (Kwiatkowska et al., 2013, Vontas J et al., 2007).

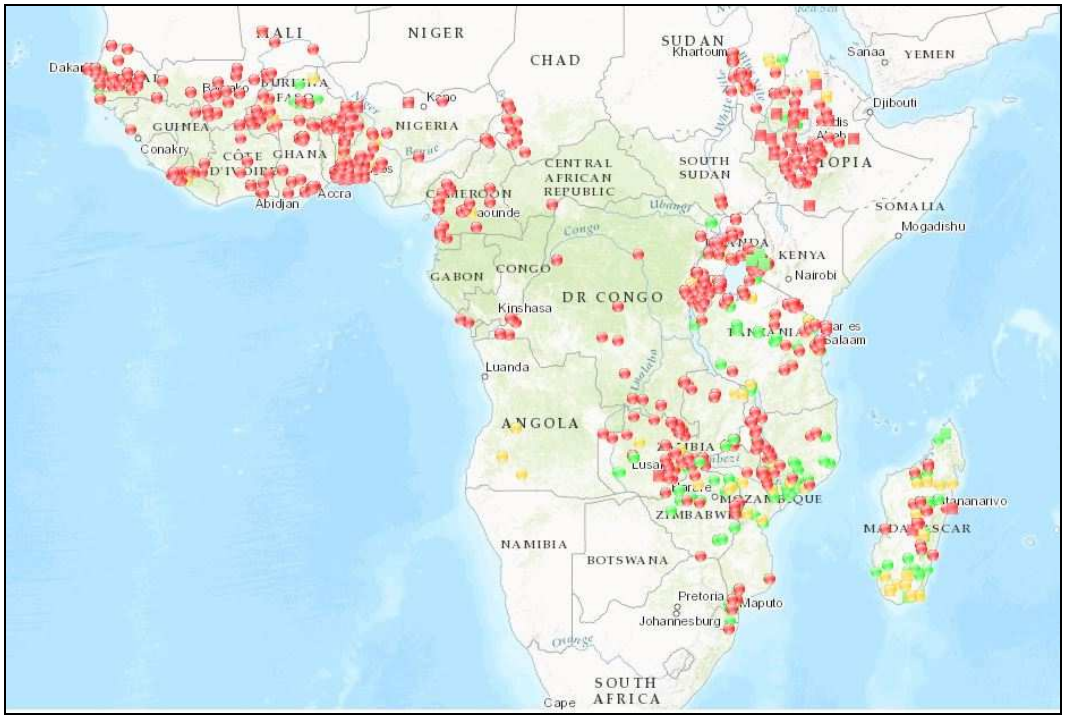

Figure 3: Geographic distribution of resistant malaria vectors in Africa as at July 2016. The data is based on published articles and unpublished reports from studies. The central Africa data is unavailable. Red dots indicate where resistance has been reported for either DDT or pyrethroids and represents either target site or metabolic resistance or both in malaria vectors. Yellow dots represent localities where resistance is suspected (according to WHO protocol where mortality is less than 98% but greater than 90%) while green dots represent those where vectors are fully susceptible. Image source: Irmapper.com: http://irmapperjavascriptwcfservice.cloudapp.net/

1.5 Insecticide resistance and vector bionomics

Though resistance is beneficial to survival in the presence of insecticides, it may have pleiotropic effects that results in longer larval development time, laying of fewer eggs with increased locomotor activity compared to susceptible mosquitoes (Brito et al., 2013). Also,

resistant populations divert enormous energy to being resistant (McCarroll and Hemingway, 2002) and remaining energy may not be sufficient for parasite growth (McCarroll et al., 2000) although this is difficult to ascertain in the wild.

Most studies that investigate parasite growth and hence transmission potential infect mosquitoes and dissect their abdomens to demonstrate and count particular development stage of parasites; oocysts for the case of malaria. Reduced number of oocysts has been used to imply reduced transmission capabilities. However, few oocyts might be an evolutionary response (Cohuet et al., 2010) on the part of the parasite to remain transmissible because fewer oocysts can still produce high number of sporozoites that infect susceptible populations (Burkot et al., 1987, Alout et al., 2013).

Experimental infection studies using wild genetically unmodified mosquitoes employing the same methods have demonstrated contrasting results where *An. arabiensis* that were homozygous and heterozygous for *kdr* had more oocyst and sporozoite stage infection (Ndiath et al., 2014) compared to susceptible vectors. Nevertheless, through such studies, it might not be easy to explicitly discern the roles that insecticide resistance play in malaria transmission.

1.5.1 Insecticide resistance and malaria transmission

The potential impact of insecticide resistance on malaria transmission has been a concern since the mid-20th century. Just after the scale-up of DDT use to control malaria during Global Malaria Eradication Campaign (GMEC), resistance developed and is one of the many reasons that led to the abandonment of the campaign (Nájera, 1999).

Causal relationship between insecticide resistance and malaria transmission is challenging to investigate (Kleinschmidt et al., 2015) and therefore many studies both theoretical (Saddler and Koella, 2015, Briët et al., 2013) and empirical (N'Guessan et al., 2007, Asidi et al., 2012) have relied on the indirect effects of resistance on malaria vectors to imply potential effect in malaria transmission and control. More direct links of insecticide resistance and malaria transmission perhaps come from select cases from South Africa, Malawi, Senegal and Bioko Island.

In South Africa, resistance to pyrethroids greatly led to rebound of malaria until the re-introduction of DDT in IRS that saw malaria reduce tremendously (Hargreaves et al., 2000). In Malawi, scaling up of pyrethroid based IRS activities did not see reductions in transmission although overall malaria cases in the country did not increase with emergence of resistance (Wondji et al., 2012). In Senegal, longitudinal analysis revealed how reducing malaria prevalence trends were negated when the frequency of *Vgsc-1014F* allele rose from 7 to 48% but this might be confounded by the shift of at risk age group that led to more malaria cases (Trape et al., 2011).

Similarly in Bioko Island, malaria transmission until 2004 was high with annual EIR over 200 up to 700 (Cano et al., 2004). Spraying of pyrethroid insecticides (deltamethrin™ and fendona™) during IRS did not have an impact on the density of resistant *An. gambiae* s.l until the second and third rounds that bendiocarb was introduced in place of the pyrethroids (Sharp et al., 2007). Although EIR was reduced in the first year, only modest but significant reductions in parasite prevalence occurred (Kleinschmidt et al., 2006).

Despite these studies showing effect of insecticide resistance, other studies have reported continued beneficial advantage of LLINs and IRS in reducing malaria transmission even in areas of insecticide resistance (Henry et al., 2005, Lindblade et al., 2015, Tokponnon et al.,

2014) (Corbel et al., 2012). Whether insecticide resistance contributes to malaria heterogeneity remains to be determined.

1.6 Malaria transmission hotspots

The impetus to eradicate malaria (Tanner and Savigny, 2008) has motivated malariologists to study, more explicitly, malaria transmission in different transmission settings to determine the factors that drive its heterogeneity (Alonso et al., 2011). As transmission falls, at least in part in response to increased control efforts, (WHO, 2013b) heterogeneity in transmission is expected to become apparent (WHO, 2007, Woolhouse et al., 1997).

Marked heterogeneity in transmission has already been documented (Bousema et al., 2010, Thomas and Lindsay, 2000) even at village level (Burkot et al., 1988, Browne et al., 2000) and in areas of overall reduced transmission like The Gambia (Mwesigwa et al., 2015, GNMCP, 2011).

Thus, transmission foci, referred to as 'hotspots' that persistently have higher malaria transmission rates (Gaudart et al., 2006, Ernst et al., 2006, Kreuels et al., 2008) than contiguous areas pose challenges to malaria control programmes. This is because they may be recalcitrant to conventional malaria control tools and they may maintain and act as new sources of infection to other areas (**Figure 4**) (Bejon et al., 2010, Macdonald, 1953).

Understanding the epidemiological factors that contribute to the creation and maintenance of these hotspots is crucial for targeted malaria control interventions geared towards malaria elimination. Indeed, in his presidential address at the Royal Society of Tropical Medicine, the late George MacDonald noted that "…*From the point of view of prevention, it would be more profitable to study the endemic reservoir and the factors which make its maintenance*

possible…strategy in prevention could then be directed not at some widely diversified overall objectives, but at the concentrated objectives of the natural foci themselves…" (MacDonald, 1965).

However, it is difficult to identify hotspots in a highly endemic area with high transmission because majority of the people are asymptomatic carriers and therefore few febrile cases/incidences are reported making identification of new infections difficult (Smith et al., 1993). On the contrary, in low transmission areas, isolated foci that continue to persist and have malaria incidences may be more readily identified as in such settings not many carry sub-patent infections (Okebe et al., 2014).

During the 1930s programme of eliminating *Anopheles gambiae* from Brazil, 'key areas/ mother foci' that acted as main source of malaria were targeted (Soper and Wilson, 1943). Similarly, eradication of *Aedes aegyptii* from the Americas also targeted 'key areas' (Soper and Wilson, 1942) indicating that such areas that were suspected to be the nucleus of the diseases was important in disease control.

Targeting hotspots may however not be beneficial in sparse settlements where houses/dwellings are further apart as was recently observed in western Kenya (Bousema et al., 2016). In that study, despite targeting control intervention at the hotspots, transmission continued and significant reductions in transmission in non-hotspots were not observed. Furthermore, presence of resistant vectors could still have perpetuated transmission in both hotspots and non-hotspots. Also, in such settings, due to heterogeneous nature of transmission several undetected smaller hotspots with different vectors may perpetuate transmission in non-hotspots.

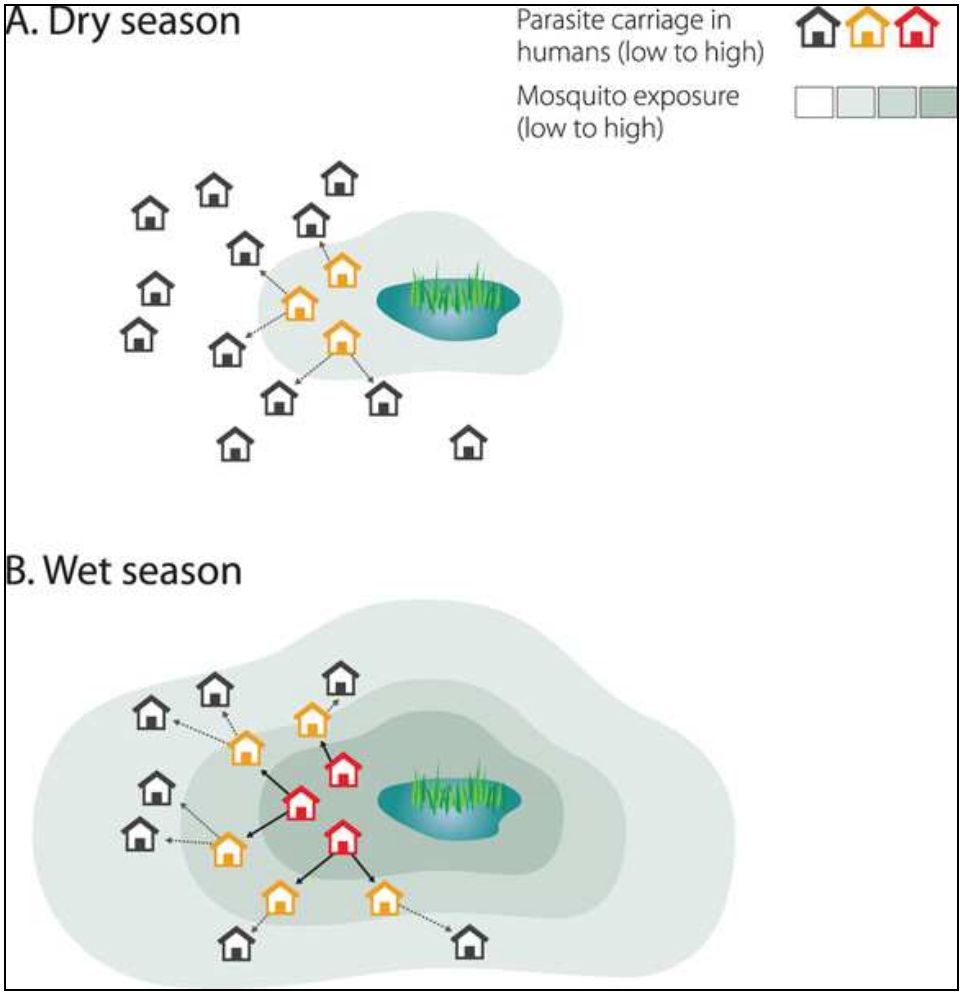

Figure 4: Hotspots of malaria transmission in the dry and wet season. Houses that are proximal to breeding habitat in the dry season (top) maintain low transmission and thus act as reservoir/hotspot of malaria and during the wet season, spreads outward to neighbouring houses (bottom). Adapted from Bousema *et al* (Bousema et al., 2012) http://www.plosmedicine.org/article/info:doi/10.1371/journal.pmed.1001165

Several factors may contribute to malaria hotspots. Variation in human attractiveness to vectors (Lindsay et al., 2000, Lindsay et al., 1993b), especially to specific malaria vectors (Knols et al., 1995), may contribute to hotspot generation if certain select individuals constantly receive infectious bites (Scott et al., 2006). The variation is brought about by differences in olfactory cues like body odour, heat and moisture that mosquitoes use to locate a vertebrate host (Mukabana et al., 2002).

Another crucial factor that could drive hotspots is the availability of hosts upon which to feed (Minakawa et al., 2002) where malaria vectors spatial distribution is dependent on host availability. Besides, those individuals who expose themselves (Geissbuhler et al., 2007) (Huho et al., 2013) to mosquito bites will have a higher likelihood to get infected.

Host genetic factors that lead to changes in the parasite's environment within the human host like having sickle cell may protect against malaria infection (Allison, 1954). Other host genetic factors may exist that result in differential susceptibility of humans to malaria parasites (Fortin et al., 2002).

The malaria parasite may modify the behaviours of the vectors to increase its transmissibility (Cohuet et al., 2010). Infected mosquitoes have been shown to continually forage on humans both in laboratory (Wekesa et al., 1992) and field (Koella and Packer, 1996) experiments increasing the probability that the pathogen is transmitted to other susceptible hosts (Koella et al., 1998). All these adaptations happen in the background of vector resistance to infection (Riehle et al., 2006) where some vectors will be more efficient transmitters than others.

Whether insecticide resistance that has spread in much of SSA including The Gambia (Ranson and Lissenden, 2016) is implicated in malaria transmission is unknown. Furthermore, whether differences in host choice for blood meal among sympatric malaria vectors like those occurring in The Gambia contribute to heterogeneity also remains undetermined.

This study was conducted in The Gambia where malaria intensity has declined over the years ever since the first reports of malaria in 1947-50 where prevalence in the coast stood at about 50%. The public health importance of malaria was only recognized after early European visitors to the capital Bathurst, present day Banjul, died of the disease (McGregor and Smith,

1952). At the time, children under the age of five were more at risk with 100% of children aged between 2 and 5 harbouring malaria parasites. Prevalence however declined with age to about 20%. In the rural village of Keneba near the coast, mortality due to malaria in children was high with only one half of children reaching the age of 5 years (Billewicz and McGregor, 1981).

In 1967 in the capital Bathurst, parasite prevalence declined to 2.5% (Harverson et al., 1968) and steady decline in malaria endemicity were reported in many settings across the country (Marsh et al., 1989, Greenwood and Pickering, 1993). Several factors like availability of drugs, knowledge about the disease, basic protection measures and urbanization could have contributed to the observed decline (Lindsay et al., 1990). Today, intense malaria control interventions have probably contributed a significant part in further reductions observed in the Gambia (Ceesay et al., 2008, Ceesay et al., 2010).

To select the study sites, two cross-sectional surveys were undertaken in the Gambia. First, a nationwide school survey was done to identify sites where antimalarial antibodies among school children were highest (Takem et al., 2013). Schools were then stratified by location and ranked by prevalence of antimalarial antibodies. In each stratum the school (index school) with the highest prevalence was chosen.

Six villages around the 'index' school were randomly selected and surveyed to identify villages with highest and lowest prevalence of malaria(Mwesigwa et al., 2015). Villages with high malaria prevalence were considered as hotspots. Hotspot was defined as a village that had higher than average malaria prevalence when compared to neighbouring villages within a region. Malaria parasite rates were monitored yearly to determine the temporal stability in prevalence between the hotspots and coldspots.

2 CHAPTER TWO: RELATIONSHIP BETWEEN INSECTICIDE RESISTANCE AND MALARIA TRANSMISSION HETEROGENEITY IN THE GAMBIA

This chapter was published in peer reviewed journal, Malaria Journal, as **"Does Vector Insecticide Resistance Contribute to Heterogeneity in Malaria Transmission in The Gambia?"**

Here, it is presented with minor modifications

Does Insecticide Resistance Contribute to Heterogeneity in Malaria Transmission in The Gambia?

Abstract

Introduction

Malaria hotspots, areas with consistently higher average transmission, may become increasingly common as malaria declines. This phenomenon, currently observed in The Gambia, can be caused by several factors, including some related to the local vectors, whose contribution is poorly understood.

Methods

Using WHO susceptibility bioassay protocol, insecticide resistance status was determined in vector populations sampled from 6 pairs of villages across The Gambia, in each pair a low and high prevalence village.

Results

Three vector species with balanced frequencies overall (23.5% *Anopheles arabiensis*, 31.2% *An. gambiae*, 43.3% *An. coluzzii* and 2.04% *An. coluzzii* × *An. gambiae* hybrids) but with broad-scale variation were detected. Even at a fine scale, significant differences in species composition were detected within village pairs. Resistance to both DDT and deltamethrin was much more common in *An. gambiae*, most markedly in the eastern part of The Gambia, partly attributable to differing frequencies of resistance mutations. The *Vgsc*-1014F target site mutation was strongly associated with both DDT (OR=256.7, (95% CI 48.6 – 6374.3, $p<0.001$) and deltamethrin survival (OR= 9.14, (95% CI 4.24 – 21.4, $p<0.001$). A second target site mutation, *Vgsc-1575Y*, which co-occurs with *Vgsc*-1014F, and a metabolic marker

of resistance, *Gste2-114T,* conferred additional survival benefits to both insecticides. DDT resistance occurred significantly more frequently in villages with high malaria prevalence (p=0.025) though this did not apply to deltamethrin resistance.

Conclusion

Whilst causality of relationships requires further investigation, variation in vector species and insecticide resistance in The Gambia is associated with malaria prevalence. In areas with heterogeneous malaria transmission, the role of the vector should be investigated to guide malaria control interventions.

2.1 Introduction

Malaria foci, referred to as 'hot spots', have persistently higher transmission rates (Gaudart et al., 2006, Ernst et al., 2006, Kreuels et al., 2008) than contiguous areas and pose challenges to malaria control programs. They may be refractory to conventional malaria control tools and may maintain and act as sources of infection to surrounding areas (Bejon et al., 2010, Macdonald, 1953). As transmission falls, partly in response to control efforts scale up, (WHO, 2013b) heterogeneity in transmission will become more apparent (WHO, 2007, Woolhouse et al., 1997). Marked heterogeneity in transmission has been documented (Bousema et al., 2010, Thomas and Lindsay, 2000) even at village level (Burkot et al., 1988, Browne et al., 2000), and in areas of overall reduced transmission like The Gambia (Mwesigwa et al., 2015, GNMCP, 2011).

Understanding the epidemiological factors that contribute to the emergence and maintenance of these hotspots is crucial for interventions aiming at malaria elimination. Local human behaviour (Stoddard et al., 2009, Klinkenberg et al., 2006, Wesolowski et al., 2012), vector behaviour (Smith et al., 1995, Gillies, 1988), environmental factors (Midega et al., 2012, Klinkenberg et al., 2008, Ijumba et al., 2002, Lindsay et al., 2003) and their interplay may give an insight into the transmission dynamics in hotspots. Malaria vector species and populations vary in space and time (Smith et al., 1995), in their anthropophily, exophily and endophily (Gillies, 1988) and, importantly, in the level of insecticide susceptibility (Santolamazza et al., 2008a).

Insecticide resistance against available insecticides has been widely reported in malaria vectors and varies in space (Edi et al., 2012, Antonio-Nkondjio et al., 2011, Dabire et al., 2007, Casimiro et al., 2006, Corbel et al., 2007, Coetzee et al., 2006, Ochomo et al., 2014).

Although a causal relationship between insecticide resistance and malaria transmission has not been shown, spatial variation in susceptibility to insecticides may contribute to the observed heterogeneity in malaria transmission (Zhou et al., 2011). Since mosquitoes resistant to insecticides survive longer than their susceptible counterparts in the presence of an insecticide, they may live long enough (N'Guessan et al., 2007) to affect malaria transmission (Molineaux et al., 1979, Hargreaves et al., 2000, Garrett-Jones and Grab, 1964). Therefore, insecticide-resistant vectors may maintain transmission (Busvine and Pal, 1969) or, where control interventions have been successful, reverse gains (Busvine, 1978, Hargreaves et al., 2000, SAMCP, 2001).

2.1.1 Malaria in The Gambia

In The Gambia, malaria transmission has decreased substantially over the last few years and it has become increasingly heterogeneous (GNMCP, 2011, WHO, 2013b, Mwesigwa et al., 2015). Malaria transmission follows the rainfall pattern, beginning after the onset of the rains and peaking between October and November. Malaria prevalence in children under the age of 5 years is approximately 4-5% nationally, though in some areas these are between 2% and 15% (Ceesay et al., 2010, GNMCP, 2011, WHO, 2013b). In the eastern Gambia, cross-sectional survey across all ages in 2012 estimated malaria prevalence at above 30%.

Malaria control, coordinated by the Gambia National Malaria Control Programme (GNMCP), largely employs Long Lasting Insecticide treated bed Nets (LLINs) and Indoor Residual Spraying (IRS) with DDT (MoH, 2012). Between 2013 and 2014, the GNMCP carried out mass distribution campaigns of Permanet® LLINS.

While cross-sectional survey across selected Gambian villages showed over 90% bed net use (Mwesigwa et al., 2015), the national LLIN usage in children under the age of 5 years stands at 60 % while that in pregnant women is only 40% (GNMCP, 2011). The difference in bed net use arise because one represent a national estimate while the cross-sectional data represent only few villages in each region.

To determine bed net use, investigators ask respondents whether they slept under a bed net the previous night. Interviewee bias to say yes might result in overestimation of the actual bed net use. This is because while collecting mosquito samples, I observed that majority of individuals retire to bed past mid night. Furthermore, at the peak of transmission, due to warm weather, villagers tend to sleep outside unprotected. This human behaviour and malaria risk will be reported elsewhere by another author (Susan Dierickx et al).

IRS with DDT has been done yearly since 2008 throughout the country except the coastal region where malaria transmission is extremely low. The first line treatment is artemether-lumefantrine; pregnant women receive sulfadoxine-pyrimethamine as intermittent preventive treatment while children 3-59 months old in Upper and Central River Regions (URR & CRR) benefit of seasonal malaria chemoprevention with amodiaquine and sulfadoxine-pyrimethamine since the 2014 transmission season.

Vector control activities carried out by the Gambia National Malaria Control Programme (GNMCP) have probably played a major role in reducing transmission (GNMCP, 2011). However, these gains may be hampered by insecticide resistance that has been recently observed in The Gambia (Tangena et al., 2013, Pinder et al., 2014). Vector species distribution varies from East to West along the River Gambia (Caputo, 2008). Four malaria vectors, *An. gambiae s.s., An. coluzzii, An. arabiensis* and *An. melas* maintain transmission. *An. melas* is mainly confined to brackish waters near the coastal region but extends up to

approximately 200 kilometres inland during the rainy season (Majambere et al., 2008, Caputo, 2008, Bryan et al., 1986). During the rainy season, the population of *An. gambiae s.s.* rises non-uniformly across the country while *An. arabiensis* and *An. coluzzii* persist longer into the dry season (Caputo, 2008).

The local dynamics of insecticide resistance may be impacted by the spatio-temporal variation in insect vectors (Reimer et al., 2005, Dabiré et al., 2008, Djègbè et al., 2011), which can result from different ecological niche preferences (Costantini et al., 2009, Gimonneau et al., 2012). In scenarios where populations are separated by ecological factors or barriers, different resistance mechanisms may develop as a result of differential selection pressure or the occurrence of different mutations. Nonetheless, occasional gene flow (Taylor et al., 2001) can transfer mutations (Clarkson et al., 2014, Edi et al., 2012, Chandre et al., 1999, Weill et al., 2000) which may rise rapidly in frequency if selected by anthropogenic activity.

As part of a larger study investigating malaria transmission dynamics in The Gambia, the distribution and patterns of phenotypic resistance and mechanisms in *An. gambiae s.l.* populations was characterized. Specifically, the hypothesis that variation in the intensity of malaria transmission may be linked with variation in insecticide resistance, mediated by differences in species composition and resistance-related mutations was examined.

2.2 Materials and methods

2.2.1 Study sites

The study was conducted in The Gambia, a West African country surrounded by Senegal except to the west that faces the Atlantic Ocean. The country is divided into five administrative regions, namely West Coast, Lower River Region – South (LRR-South), Lower River Region – North (LRR-North), Central River Region (CRR) and Upper River Region (URR) (**Figure 5**). For purposes of this study and the overall study investigating transmission dynamics, URR was subdivided into URR-North and South to form a total of 6 regions. Six pairs of villages, one pair per region, were selected on the basis of malaria prevalence determined by a nationwide cross-sectional survey (Mwesigwa et al., 2015) (**Figure 5**). In each pair, the village with the highest prevalence and that with the lowest prevalence were included (**Appendix 2**). For all pairs there was a significant difference in infection prevalence with the exception of villages G and H in the CRR.

The Gambia has one rainy season from June through October diminishing in November. The mean daily temperature varies between 25 and 40° C. The country is primarily low altitude with seasonal flooding; it lies in the open and flat woodland Savannah belt and riverine swamps are common towards the western part of the country (Majambere, 2007, Caputo, 2008). The sea mixes with the river and during the rainy season, brackish waters mixes further inland up to approximately 200 kilometres into the river. Rice paddies are common on the margins of the river, especially in the CRR. Towards the east, cereal crop farming is practised.

2.2.2 Study design

Mosquitoes were sampled between July and October 2013 from the 12 villages. Larval collections were conducted within a 2 kilometre radius of the centre of the villages and transported to a central insectary in Wali Kunda (13° 34' N, 14° 55' W) for rearing and testing. Blood fed adult female collections were performed in villages that had few or no observable breeding habitats. Blood-fed anophelines were transferred to the insectary on the same day of collection where they were kept in individual paper cups containing moistened Whatman filter papers to induce egg laying. The females were also provided with 10% glucose solution on a cotton wool plug. Eggs from blood-fed mosquitoes from one village were grouped together and allowed to mix randomly.

Mosquitoes, including an insecticide-susceptible colony from Yaoundé, Cameroon, were reared under similar conditions. Larvae were fed on Tetramin® (Tetramin gmbH Germany) fish food and maintained at 28°C and 80% humidity. Upon emergence, adult mosquitoes were provided with 10% glucose. The WHO protocol (WHO, 2013a) on insecticide susceptibility tube assays was used to assay phenotypic resistance.

Three to five day old mosquitoes in groups of 20-25 were exposed for an hour to either 4% DDT or 0.05% deltamethrin impregnated papers (WHO, 2013a). These two insecticides were chosen because the GNMCP distributes deltamethrin-impregnated LLINs (Permanet®) and uses DDT in IRS campaigns. A total of 1,005 field collected *Anopheles gambiae* s.l. were tested. Mortality in the control group was always less than 5%. After the phenotypic assays, all mosquitoes tested were stored in 1.5ml Eppendorf tubes with silica gel and transported to the MRC Fajara for species identification and screening for insecticide resistance genetic markers.

2.2.2.1 Laboratory processing

DNA from all mosquitoes was extracted using a Qiagen kit according to manufacturer's protocol (Qiagen mini kit manual extraction method). Two polymerase chain reaction protocols (Scott et al., 1993, Santolamazza et al., 2008b) were used to identify the *Anopheles gambiae s.l.* to species level. Scott's protocol was used to identify *An. gambiae s.s.*, *An. arabiensis, An. melas* while the SINE-PCR (Santolamazza et al., 2008b) protocol was used to further distinguish the *An. gambiae s.s.*, from *An. coluzzii* and *An. arabiensis* simultaneously.

All mosquitoes tested in the insecticide resistance bioassay were genotyped, using TaqMan assays (Bass et al., 2007b, Bass et al., 2010, Jones et al., 2012a, Mitchell et al., 2014), for five markers of insecticide resistance, namely the *Vgsc*-1014F and *Vgsc-1014S* mutations in the voltage gated sodium channel gene that confer resistance to DDT/pyrethroids, *Vgsc-1575Y* which enhances action of the *1014F* mutation, *Gste2-114T* which has been associated with metabolic resistance to DDT, and *Ace1-119S* which is associated with resistance to carbamates and organophosphates in DDT resistant mosquitoes (Jones et al., 2012a).

2.2.3 Statistics

Statistical analysis was done using R statistical package (R version 3.1.2, 2014). Tests of differences of proportions were done to investigate differences in vector populations. Fisher's test was used to determine differences in species composition using an online algorithm (http://in-silico.net/tools/statistics/fisher_exact_test). Pearson's chi-squared test for proportions was used to test for differences in mortality between species and villages. Non-parametric tests were used to investigate differences in mortality to insecticides within pairs of study villages and, more generally, geographic variation in insecticide resistance.

Differences between individual proportions were assessed using Marascuilo's procedure (Marascuilo, 1966).

Binomial confidence intervals (Newcombe, 1998b, Wilson, 1927) were calculated for species distribution and mortality to insecticides. Odds ratios were used to estimate the effect size of DNA marker assays in relation to resistance phenotype. Further, General Linear Models (GLM) with logit link function for a binomial dependent variable was used to model the effect of different mutations, sampling site and species on mortality. In addition, GLM was used to investigate the interaction between DNA resistance markers in conferring survivorship to mosquito samples.

Differences in mortality trend was determined by first grouping villages into 3 regions, Eastern, Central and Western villages according to ecological zones identified by Caputo *et al* (Caputo, 2008). Western villages consisted of A- Bessi, B- Ndemban Tenda, C -Chogen Wellingara and D - Yallal Ba, central villages were E - Sinchu Njengudi, F - Dongoro Ba, G - Sare Seedy and H – Ngedden, and eastern villages were J-Njaiyel, K-Madina Samako, L-Sare Wuro and M-Gunjur Koto.

2.2.4 Ethical Clearance

This study was approved by Medical Research Council Unit (MRC) scientific coordinating committee and ethical clearance obtained from The Gambia Government / MRC Joint Ethics committee. Informed oral consent was obtained during village sensitization meetings.

2.3 Results

In the 2013 collection season, 1,005 mosquitoes were tested using the WHO tube bioassay protocol (WHO, 2013a); 508 against 4% DDT, 497 against 0.05% deltamethrin. *An. gambiae* s.l. was sampled from all but two villages, Madina Samako and Chogen Wellingara (**Figure 6**). Three members of the *Anopheles gambiae* complex were identified: *An. gambiae s.s., An. arabiensis,* and *An coluzzii* together with some *An. gambiae* s.s. x *An. coluzzii* hybrids (**Figure 5**). Of the paired study villages, vector composition could only be compared in four pairs because the 2 remaining pairs lacked mosquitoes in one or both of the constituent villages. In three of the four village pairs, species composition varied between high and low transmission village pairs (**Figure 5**).

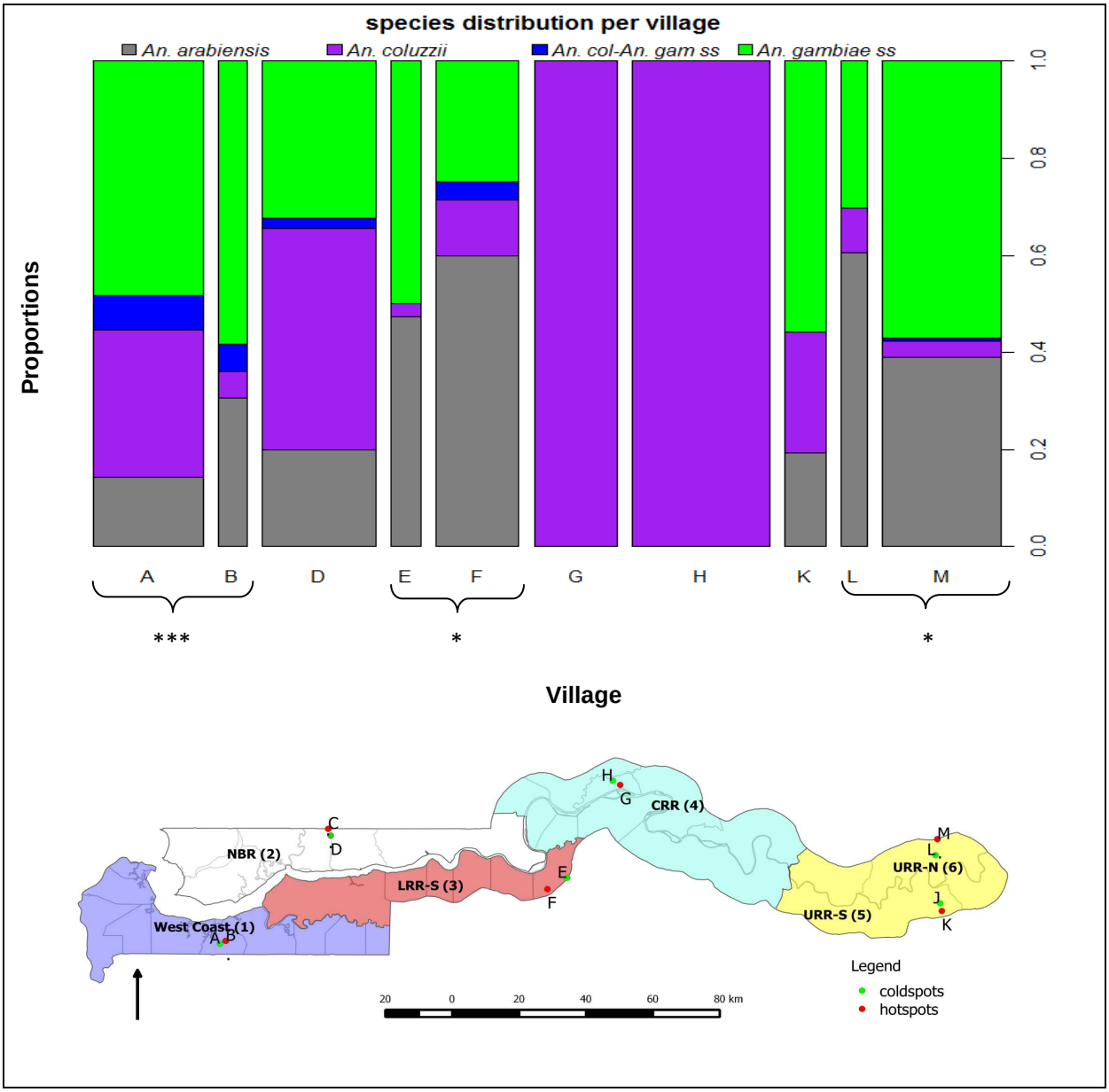

Figure 5: Graph showing the relative proportion of malaria vectors across study sites in The Gambia. Width of bars represents sample sizes (**see Appendix 3**). Asterisks indicated significant differences in species diversity between village pairs assessed using Fisher exact tests (where collections and >1 species collected, permitted test); ***P<0.001; *P<0.05 Below it is administrative map of The Gambia showing study sites/villages in 6 geographic regions (numbered). 1 - West Coast, LRR-N (Lower River Region-North), LRR-S (Lower River Region-South), CRR-N (Central River Region-North), URR- S/N (Upper River Region- North/South). The green dots indicate low malaria prevalence while red dots – high malaria prevalence villages. A- Bessi, B-Ndemban Tenda, C-Chogen Wellingara, D-Yallal Ba, E-Sinchu Njengudi, F-Dongoro Ba, G-Sare Seedy, H-Ngedden, K-Madina Samako, J-Njaiyel, L-Sare Wuro, M-Gunjur Koto.

2.3.1 Phenotypic resistance to DDT and Deltamethrin in a WHO bioassay

There were significant interspecies differences in the 24-hour post-exposure mortality to DDT and deltamethrin. For DDT, resistance was most pronounced in *An. gambiae s.s.*, with only 37% mortality (95% CI 29 – 46%), compared to the other four species (Pearson Chi-squared test, $\chi^2 = 194$, df= 3, $p < 0.001$) (**Figure 6**). Further analysis showed significant differences in mortality except between *An. arabiensis* and *An. coluzzii* (**Appendix 4)**. There were also significant differences in mortality between species following deltamethrin exposure (Pearson's Chi-squared test, $\chi^2 = 44.94$, df= 3, $p < 0.001$). A significant difference in mortality was only observed when species were compared to *An gambiae* s.s., with the exception of *An. coluzzii* × *An. gambiae s.s* hybrids (**Appendix 4**). There was a significant correlation between DDT and deltamethrin mortality (Kendall's correlation weighted by village, tau=0.61, p = 0.02), indicating that, *An. gambiae s.s.* was likely to be resistant to both insecticides.

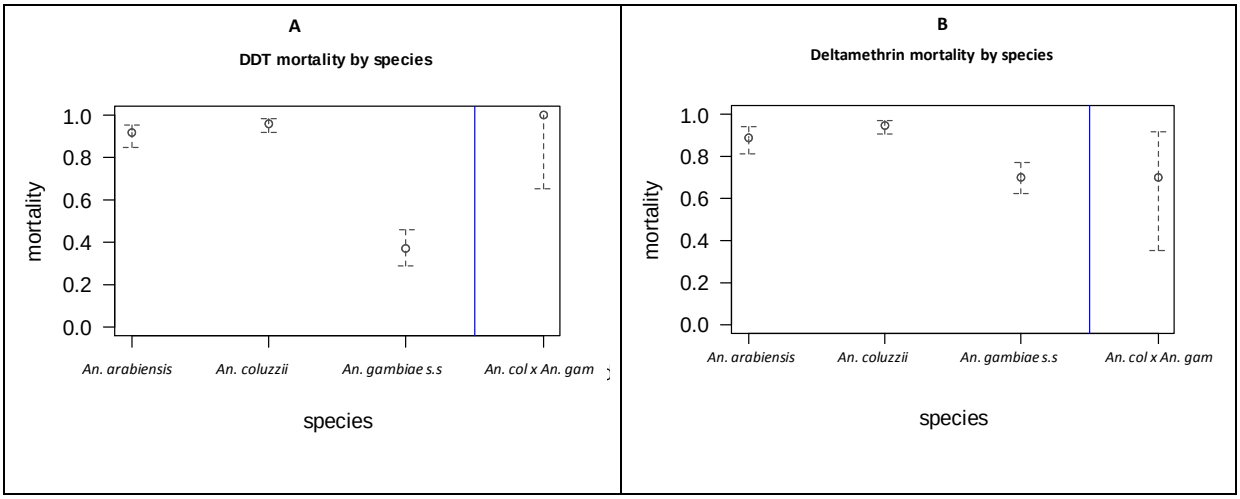

Figure 6: Species-specific mortality to DDT (panel A) and deltamethrin (panel B) across all study villages.

There was variability in inter species mortality within and between villages for deltamethrin (χ^2= 9.14, p=0.03) while for DDT, variability was of borderline significance (χ^2=7.78, p =0.05). *Anopheles gambiae s.s.* from the east were more resistant than those from the western part of the country (**Appendix 5**). DDT mortality tended to decrease from west to east, starting from Sinchu Njengudi (E). For deltamethrin, there was a similar trend though reduced mortality was mainly in Madina Samako (K), Sare Wuro (L) and Gunjur Koto (M) (**Figure 7**).

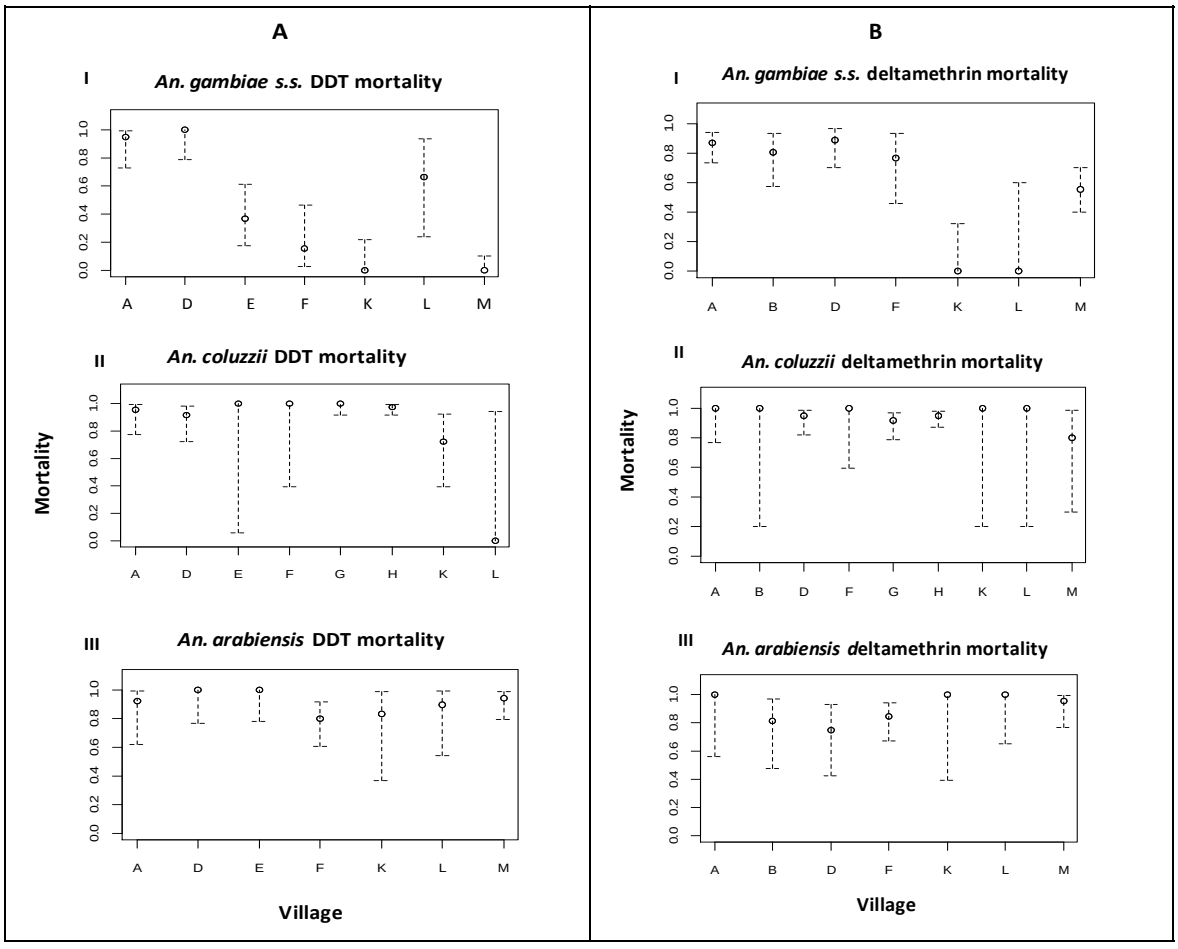

Figure 7: Mortality by species and village (sampling site). Panel A - mortality against DDT, panel B - mortality against deltamethrin. Villages are labelled as in Figure 1

2.3.2 Resistance association of DNA markers

The frequency of resistance alleles for various markers varied among species, with the *Vgsc – 1014F* mutation being most common in *An. gambiae s.s.* (**Table 2**), which had in this species a highly significant association with resistance to both DDT and deltamethrin. It was not possible to conduct these tests on the other species due to the low frequency of the *Vgsc*-1014F resistance mutation and the high mortality.

In *An. gambiae s.s.*, survival of the *Gste2-114T* carriers was also significantly increased for DDT and (unexpectedly) for deltamethrin (**Table 3**). For *An. coluzzii*, there was no significant association between *Gste2-114T* and DDT resistance although a significant negative effect was observed for deltamethrin (**Table 3**).

Table 2: Allele frequency, in percentage expressed as proportions (allele/total number of alleles), of insecticide resistance mutations of malaria vectors in The Gambia

Allele frequency (with 95% confidence intervals) of molecular resistance markers by species					
Species	***Vgsc - 1014F***	***Vgsc - 1014S***	***Vgsc - 1575Y***	***Gste2-114T***	***Ace-1 119S***
An. gambiae s.s.	0.51 (0.45 - 0.55)	0.004 (8.56^{-5} – 0.01)	0.13 (0.1 – 0.16)	0.097 (0.075 – 0.12)	0.003 (5.68^{-4} – 0.01)
An. arabiensis	0.05 (0.02 – 0.06)	0.14 (0.1 – 0.17)	0.002 (1.13^{-4} – 0.01)	0.017 (0.008 – 0.04)	0 (0 - 0.1)
An. coluzzii	0.0012 (6.16^{-5} – 7.62^{-3})	0 (0 – 0.01)	0.0012 (6.16^{-5} – 0.01)	0.67 (0.67 – 0.74)	0 (0 – 0.01)
***An. coluzzii x An. gambiae* hybrid**	0.15 (0.063 – 0.31)	0 (0 - 0.11)	0.08 (0.02 – 0.22)	0 (0 - 0.11)	0 (0 - 0.11)

Table 3: Odds ratios of *An. gambiae s.s.* and *An. coluzzii* mutants surviving an insecticide exposure for each insecticide resistance marker.

				95% confidence intervals		
species	**Insecticide**	**marker**	**Odds Ratio**	**Lower**	**Upper**	**P**
An gambiae ss	DDT	Kdr	253.74	48.07	6302.05	< 0.001
		GSTe2	3.4	1.43	9.18	0.01
	deltamethrin	Kdr	8.37	3.99	18.47	< 0.001
		GSTe2	3.4	1.175	10.29	0.02
An coluzzii	DDT	GSTe2	1.5	0.34	11.35	0.72
	deltamethrin	GSTe2	0.23	0.06	0.78	0.02

kdr refers to *Vgsc-1014F* mutation

Species, village and *Vgsc*-1014F *e*xplained significant variation in mortalities to both insecticides, though *Gste2-114T, Vgsc-1575Y* and interactions between markers were not significant (**Table 4**). Because of the absence of a sufficient number of survivors carrying resistance mutations in other species, other than *An. gambiae s.s.,* interaction between species and markers was not included in the model. A backward stepwise logistic regression therefore excluded *Gste2-114T* and *Vgsc- 1575Y* in the final model (**Appendix 6 and Appendix 7**). All the molecular markers screened in this study played a role in insecticide resistance but their effect was masked by the presence of the *Vgsc*-1014F mutation in captured *An. gambiae* s.s. which was a strong predictor of insecticide resistance.

Table 4: The effects of village, species and resistance markers on mortality of mosquitoes to DDT and deltamethrin using GLM.

	Factor	Df	Deviance	Residual Df	Residual Deviance	P
DDT						
	species	5	190.56	490	331.83	< 0.001
	village	8	114.2	482	217.62	< 0.001
	kdr	5	51.04	477	166.58	< 0.001
	1575Y	2	1.05	475	165.53	0.59
	gste2	2	1.8	473	163.73	0.41
	kdr:1575Y	1	0.86	472	162.87	0.35
	kdr:gste2	4	4.33	468	158.55	0.36
	1575Y:gste2	2	1.13E-08	466	158.55	1
Deltamethrin						
	species	5	50.46	482	371.69	< 0.001
	village	8	34.45	474	337.24	< 0.001
	kdr	5	28.22	469	309.02	< 0.001
	1575Y	2	3.13	467	305.9	0.21
	gste2	2	4.26	465	301.63	0.12
	kdr:1575Y	1	0.09	464	301.54	0.76
	kdr:gste2	3	4.07	461	297.47	0.25
	1575Y:gste2	3	0.86	458	296.61	0.83

Df refer to degrees of freedom, Deviance-variation explained by a factor. Residual deviance-variation unexplained by factors.

2.3.3 Insecticide Resistance and malaria transmission

For *An. gambiae s.s.,* mortality to DDT and deltamethrin was compared between high and low malaria prevalence villages. The unpaired Wilcoxon sum rank test was used because some villages did not have mortality data. DDT mortality for *Anopheles gambiae s.s.* was significantly lower in high prevalence than low prevalence villages (Wilcoxon W=0, p=0.03). There was no observed difference in *An. gambiae s.s.* mortality to deltamethrin between high and low prevalence villages (W= 3.5, p= 0.24) or for any of the other species for both insecticides.

2.4 Discussion

Phenotypic resistance to DDT and deltamethrin was found mainly in *An. gambiae s.s.* and was more common in eastern Gambia where malaria transmission is significantly higher than in the western regions (Mwesigwa et al., 2015, Thomson et al., 1994, Okebe et al., 2014), suggesting a link between insecticide resistance and observed malaria prevalence. Previous studies exploring the association between insecticide resistance and malaria endemicity have produced contrasting results, with some reporting no effect (Henry et al., 2005, Wondji et al., 2012, Tokponnon et al., 2014, Lindblade et al., 2015) while others suggesting otherwise (Zhou et al., 2011).

In neighbouring Senegal (Trape et al., 2011) and in South Africa (Hargreaves et al., 2000), following successful malaria control, increasing insecticide resistance coincided with higher incidence of clinical malaria. Nevertheless, proving a causal relationship between insecticide resistance and malaria transmission is extremely difficult (Kleinschmidt et al., 2015).

Similar to earlier studies (Thomson et al., 1994, Jawara et al., 2008, Lindsay et al., 1993a), three malaria vectors, namely *An. gambiae s.s.*, *An. coluzzii* and *An. arabiensis*, were observed across the country and in different proportions, in addition to a few hybrids of *An. gambiae s.s.* and *An. coluzzii*. *Anopheles melas*, known to breed in brackish water and usually found in western Gambia (Majambere et al., 2008, Caputo, 2008, Bryan et al., 1982), was not collected. This may have been due to the inadequate rearing methods employed in the insectary.

Mosquito species vary from start to end of transmission season following rainfall pattern and therefore the time of collection might confound insecticide susceptibility tests when one time point is used to estimate insecticide susceptibility. However, in this study since the objective

was to document resistance levels in all species, susceptibility of specific species to insecticides was not expected to vary within season.

The extreme interspecific differences observed in insecticide resistance status and frequency of mutations among them suggests that the involvement of insecticide resistance in malaria heterogeneity would be conditional on the vector species composition. This may help explaining the differences in insecticide susceptibility estimates reported by two previous studies in eastern Gambia. In one study done in 2010, (Pinder et al., 2014), *An gambiae s.l* susceptibility to DDT and pyrethroids was about 90% while in 2011 in a village of the same region, susceptibility to the same insecticides was only 50% (Tangena et al., 2013). Such differences may be explained by the composition of the mosquito population tested. Indeed, in 2010, 70% of all anophelines were *An. arabiensis,* while in 2011 this species represented only 42% of all mosquitoes tested. Therefore, the high proportion of *An. arabiensis* may have concealed resistance in *An. gambiae s.s*.

2.4.1 Mechanisms of resistance

In *An. gambiae s.s.,* there was a clear association between the *Vgsc*-1014F mutation and phenotypic resistance, indicating that in The Gambia this is a very effective predictor of DDT and pyrethroids resistance. The *Vgsc-1575Y* and *Gste2-114T* markers had modest effects in conferring phenotypic resistance. Though in *An. gambiae s.s* and *An. arabiensis* the *Vgsc-1014S* mutation did not seem to be linked to phenotypic resistance, its low frequency limited statistical power. As in Uganda (Mawejje et al., 2013), few samples had both serine and phenylalanine mutations though carriers were also resistant to DDT. Given the low frequency of co-occurrence, it is not possible to establish whether carriage of both mutations confer an advantage, though this may be the case, at least compared to serine alone (Reimer et al., 2008).

2.4.2 Population subdivision

The different insecticide resistance profile between eastern and western Gambia raises important questions about the drivers and stability of this heterogeneity. The GNMCP has distributed LLINs across the country since 2003 and sprayed houses yearly with DDT since 2008 (GNMCP, 2011), though only intermittently in the urban west coast region because of the lower malaria transmission. IRS has been carried out in all study villages so that DDT selection pressure should have been uniform. Nevertheless, intense DDT use in a community trial investigating the additional benefits of IRS with DDT to LLIN may have increased insecticide resistance pressure (Pinder et al., 2014, Pinder et al., 2011) though it looks as if resistance was already present before its implementation (Tangena *et al* , 2013).

The high survival rates in *An. gambiae s.s.* from eastern Gambia, probably due to the high frequency of the *Vgsc*-1014F allele, suggests interbreeding with the resistant inland populations from Senegal. With no history of carbamate and/or organophosphate use for public health in The Gambia, it is interesting to note that the two mosquitoes that had a carbamate/organophosphate resistance marker, *Ace-1,* were sampled from a village that is approximately 70 kilometres from Guinguineo district, Senegal, where resistance to bendiocarb has been reported (SNMCP, 2011, NIANG, 2014), (President's Malaria Initiative, Senegal Report, unpublished, http://irmapperjavascriptwcfservice.cloudapp.net/), possibly linked to intense IRS campaigns with bendiocarb between 2008 and 2013. Investigation on the connectivity between Gambian and Senegalese *An. gambiae* populations is currently underway.

Host seeking/foraging and resting behaviour of mosquitoes have been shown to play a role in the development of insecticide resistance (Padonou et al., 2011, Kloke et al., 2011). In The

Gambia, the lack of detailed information on the behaviour of the sympatric malaria vectors limits proper insights into the causes of resistance in the eastern populations. Endophagy of *An. gambiae s.s.* may increase their exposure to insecticides, favouring the development of resistance (Stump et al., 2004, de Zulueta, 1959). Conversely, exophagy of *An. arabiensis* (Lindsay et al., 1993a) could play a role in the low levels of resistance observed in this species. However, in Senegal, where no difference in biting and host seeking behaviour were found (Diatta et al., 1998) until recently (Ndiath, 2014), resistance has been reported mainly in *An. gambiae s.s.* and to a lesser extent in *An. arabiensis* and *An. coluzzii* (Santolamazza et al., 2008a, Ndiath, 2014).

In conclusion, insecticide resistance, which varies by species, seems to be associated to malaria endemicity. Indeed, in eastern Gambia both insecticide resistance and malaria transmission are higher than in the rest of the country. The vector population is also extremely heterogeneous, underpinning the need for national malaria control programmes to continually monitor, as extensively as possible, the status of insecticide resistance to guide malaria control practices.

3 CHAPTER 3: TEMPORAL DYNAMICS OF INSECTICIDE RESISTANCE AND BLOOD MEAL CHOICE OF MALARIA VECTORS IN DIFFERENT MALARIA TRANSMISSION SETTINGS OF THE GAMBIA

Temporal dynamics of insecticide resistance and blood meal choice of malaria vectors in different malaria transmission settings of The Gambia

Abstract

The variability of species composition, their feeding habits and their insecticide resistance status may contribute to malaria transmission heterogeneity. The temporal patterns of insecticide resistance and blood feeding behaviour were investigated in several sites of varying malaria transmission in The Gambia.

Methods

Malaria vectors were collected during the 2013 and 2014 transmission seasons across The Gambia using two methods; larval sampling for resistance phenotyping against DDT and deltamethrin and adult sampling with Centres for Disease Control miniature light traps (CDC-LT) to determine malaria vectors' blood meal choices.

Results

The three species sampled remained the same in 2013 and 2014, and though there were statistically significant differences in the relative species composition in the study villages between years, similarity indices suggested variations were generally quite minor. Bioassay mortality to DDT and deltamethrin varied spatially in both years and mortality was slightly, but significantly, higher in the second year. Insecticide resistance was mainly confined to *Anopheles gambiae* s.s. and there was a trend of reduced insecticide susceptibility from west to east for all insecticides. The prevalence of *Vgsc*-1014F in *An. gambiae* s.s. in eastern Gambia and *Gste2-114T* in *An. coluzzii* is approaching fixation. The frequency of *Ace-1-119S* mutation in eastern *An. gambiae* s.s. rose from 1% in 2013 to 7% in 2014 (χ^2=11.6, p =0.001). First recent identification of *Anopheles funestus* was made in two villages in central

Gambia. All malaria vectors identified fed mainly on humans with the exception of *An. funestus* that fed mainly on cows and other animals (χ^2=162.8, p <0.001).

Conclusion

Humans were the preferred vertebrate host for *An. gambiae* s.l but not *An. funestus* that preferred cows. Little precipitation in the second year hampered sampling of malaria vectors and could have resulted in the generally few anophelines sampled in that year. The broad pattern of geographical differences in insecticide resistance to DDT and deltamethrin in *An. gambiae s.s.* remained stable. The insecticide resistance observed within *An. gambiae* s.s. of The Gambia was mainly caused by *Vgsc*-1014F and partly *Vgsc-1575Y* and *Gste2*-114T. The frequency of *Vgsc*-1014F is approaching fixation in *An. gambiae* s.s. in the east. Gains in vector control may be limited if selection for *Ace-1-119S* continue following scale–up of bendiocarb use in IRS. The GNMCP may therefore benefit from continual monitoring of insecticide resistance and to adopt World Health Organization's plan for the management of insecticide resistance in malaria vectors.

3.1 Introduction

Intensive use of DDT and pyrethroids for the control of malaria vectors exerts enormous pressure (Lynd et al., 2010), leading to the selection of mutations conferring resistance to those insecticides. In addition, following high coverage of Long Lasting Insecticidal bed Nets (LLINs) and Indoor Residual Spraying (IRS), vectors may change their host choice for a blood meal (Quiñones et al., 2000) or biting patterns (Bayoh et al., 2010, Russell et al., 2011, Sougoufara et al., 2014, Yohannes and Boelee, 2012). Although humans still remain the preferred vertebrate host for a blood meal, plasticity in the choice of other vertebrates has been documented (Lefevre et al., 2009).

As a resistance mutation spreads at different rates between and among species, variability in mortality to various insecticides becomes evident both in space and time (Chapter one, (Mathias et al., 2011, Edi et al., 2014a)).Temporally, the frequency of insecticide resistance alleles are known to vary where this index randomly fluctuate hence affecting the phenotypic characteristics of the populations that carry them. However, despite this variability in resistance traits, if the allele is beneficial and thus being selected for in the population, the frequency will ultimately rise and become fixed in the population (Lynd et al., 2010) (Ochomo et al., 2013) but not always (Badolo et al., 2012). Consequently, because of the variability in local composition of malaria vectors, their different insecticide profiles and blood feeding behaviours, malaria transmission could be impacted differentially.

Malaria heterogeneity consists of geographically limited spots of higher transmission than the surroundings, also labelled as hot spots (Bousema et al., 2012). Such heterogeneity may be supported by a variety of factors which include also blood meal choice of local malaria vectors; if they feed predominantly on humans they would contribute to transmission more substantially than those feeding predominantly on other animals (Killeen et al., 2001, Koella,

1991, Dadzie et al., 2013). In addition, vectors feeding predominantly on humans may have multiple blood meals during a single gonotrophic cycle, with some people more bitten than others (Scott et al., 2006, Norris et al., 2010, Koella et al., 1998); it is unclear whether this was the reason for the non-homogeneous malaria risk observed in Senegalese (Trape et al., 2002) and Kenyan (Mwangi et al., 2008) children. Nonetheless, *Plasmodium*-infected mosquitoes may have multiple feedings (Koella et al., 1998) and if these target a few individuals, then the latter would be more at risk for malaria infection and/or disease.

As malaria transmission hotspots seem to be stable over time, probably the causative factors are also stable. As insecticide resistance and blood meal choice may be among these factors, their temporal variation was investigated in different transmission settings across the Gambia in the 2013 and 2014 transmission seasons.

3.2 Methods

3.2.1 Study site

The study was carried out in twelve Gambian villages spread across 6 study regions as earlier identified (Mwesigwa et al., 2015). Detailed description of study villages has been done elsewhere (Chapter two; (Opondo et al., 2016)). Briefly, twelve villages spread across administrative regions of The Gambia were selected for this study. Region 5 Upper River Region (URR) was split into two regions URR South and North banks to make a total of six study regions. A pair of villages (one representing high and the other low malaria prevalence) were chosen from each of the regions to make a total of twelve villages. Beginning with western most regions, the regions were numbered from one to six (**Figure 8**). Western villages consisted of villages A, B, C and D while eastern villages were J, K, L and M (**Figure 8**). The locations of the villages were mapped using hand held global positioning system (GPS) (Garmin eTrex® 10) and overlaid on Gambian Map in Quantum Geographical Information System (QGIS version 2.8.2).

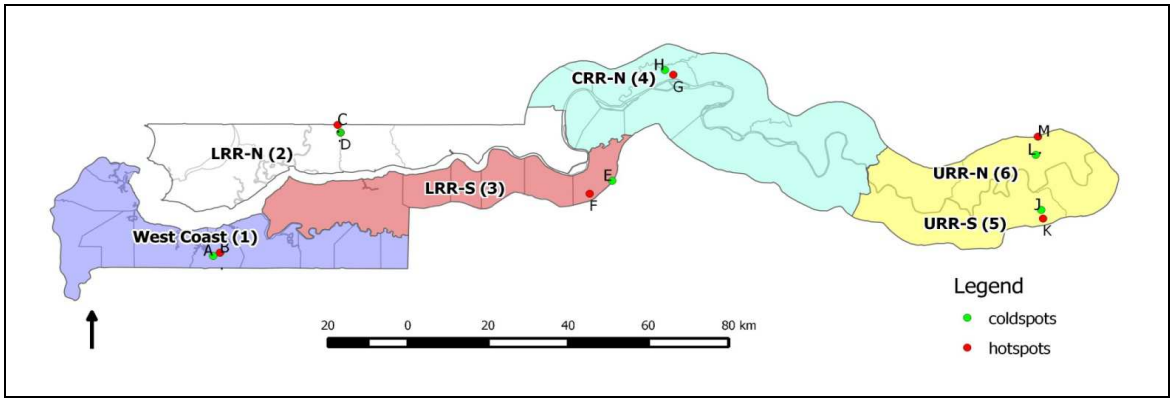

Figure 8: Administrative map of The Gambia showing study sites/villages in 6 geographic regions (numbered). 1 - West Coast, LRR-N (Lower River Region-North), LRR-S (Lower River Region-South), CRR-N (Central River Region-North), URR- S/N (Upper River Region- North/South). The green dots indicate low malaria sero - prevalence while red dots – high malaria sero-prevalence villages. A- Bessi, B-Ndemban Tenda, C-Chogen Wellingara, D-Yallal Ba, E-Sinchu Njengudi, F-Dongoro Ba, G-Sare Seedy, H-Ngedden, K-Madina Samako, J-Njaiyel, L-Sare Wuro, M-Gunjur Koto

3.2.2 Mosquito collection

3.2.2.1 For insecticide resistance phenotyping

Larvae and adult mosquitoes could be collected in all villages except Chogen Wellingara and Njaiyel in 2013 and Njaiyel and Madina Samako in 2014. Because of the paucity of malaria vectors, to increase the number of mosquitoes caught/sampled for use in the WHO insecticide resistance bioassay tube test, artificial breeding habitats were used to lure adult mosquitoes to lay eggs that subsequently developed to larvae (**Figure 9**). All sampled larvae were reared as described in chapter 2 (Opondo et al., 2016) in the insectary at the Medical Research Council (MRC) field site in Wali Kunda. Upon emergence, adults aged between 3-5 days old in groups of 20-25 were assayed for phenotypic resistance using the WHO bioassay tube test protocol (WHO 2013). All assayed mosquitoes, dead or alive, were stored in individual

Eppendorf tubes with silica gel and transported to the MRC laboratory in Fajara for molecular analysis.

Figure 9: Images showing field workers and I collecting larvae from natural breeding habitat (photo A) and artificial breeding habitat (photo B). Photo C shows WHO tubes used for insecticide resistance phenotypic assays

3.2.2.2 For determination of blood meal host choice

Within the 12 villages, from July to December, mosquitoes were collected once every month in twelve different houses. For standard comparisons, CDC-LT was only set up in single houses with one room having an individual male adult sleeper at night. The trap would be set up at 1900 hours and dismantled at 0700 the following morning after collecting trapped mosquitoes. Between village pairs, because of logistical challenges, 6 traps would be put in

one village in one night and the following night in the other member. This would be repeated twice to make a total of twelve light trap catches per village per month.

Blood meal specimens sampled using CDC-LT were then identified morphologically in four locations; Njamba Kunda Hospital laboratories, Wali Kunda and Basse field sites and make-shift entomology laboratory in Bessi. Non-vector species were identified, recorded and then discarded. Anophelines were sorted on the basis of the gonotrophic stage where unfed and semi gravid were each stored wholly in bar-coded Eppendorf tubes. Blood fed anophelines had their abdomen separated from the head and thorax and both stored in two Eppendorf tubes having identical barcode. These were transported to Fajara for further molecular processing.

Identification of *An. gambiae* s.l. and *An. funestus* sibling species was done using polymerase chain reactions (PCR) specific to *An. gambiae s.l* (Scott et al., 1993, Santolamazza et al., 2008b) and *An. funestus* (Koekemoer et al., 2002) while Kent *et al* PCR protocol (Kent and Norris, 2005) was used to identify the blood meal choice. All phenotyped and blood fed *An. gambiae s.l.* samples were screened for known molecular markers of insecticide resistance, *Vgsc*-1014F*/ 1014S* and *Vgsc-1575Y, Gste2-114T* and *Ace-1-119S* using Taqman's PCR (Bass et al., 2007a, Bass et al., 2010, Jones et al., 2012a, Mitchell et al., 2014).

3.2.3 Statistical analysis

Data was analyzed in R statistical software (R version 3.1.2, 2014). Species composition was analyzed using two similarity indices, Euclidean (Borg and Groenen, 2005) and Yue and Clayton (Yue and Clayton, 2005). Using Generalized Linear Mixed Model (GLMM) with binomial logit link function, mortality to either DDT or deltamethrin was modelled as a

function of species, insecticide, *Vgsc*-1014F/*S*, *Gste2-114T*, *Vgsc-1575Y*, region and year as fixed effects and village as random effect variable to understand the spatio-temporal dynamics of resistance.

Trend in human biting was analyzed using chi-squared test for trends in proportions. Chi-squared test was used to investigate differences in blood meal choice between and among species. Paired Wilcoxon test was used to test whether individual species in villages classified as having higher malaria prevalence were more likely to feed on humans than those in low transmission villages. Fisher's exact test was used to investigate the effect of resistance on host choice. Pearson's chi-squared test for proportions was used to test differences in proportions.

3.2.4 Ethical clearance

This study was approved by MRC Scientific Coordinating Committee and ethical clearance obtained from The Gambia Government/MRC Joint Ethics Committee. Communal informed consent was obtained from village heads and inhabitants during community sensitization activities prior to commencement of the project. A further written and signed informed consent was obtained from house owners where CDC-LTs were placed.

3.3 Results

In 2013 and 2014, a total of 2045 adult *An. gambiae* s.l. were successfully reared to the WHO recommended age of 3-5 days old and tested for phenotypic resistance to DDT and deltamethrin (WHO, 2013a) (for 2014 data See **Appendix 8**). Indoor catches using CDC-LT yielded a total of 714 blood fed anophelines from the 12 villages in 2013 while in 2014 only 93 blood fed anophelines were sampled due to little rains that greatly affected mosquito density. Detailed species distribution and composition as sampled by CDC-LT will be described elsewhere (Jawara *et al* in preparation).

Anopheles funestus, identified as *An. funestus* s.s was sampled for the first time in routine surveillance although it is known to be present in The Gambia (Gillies and De Mellion, 1968). Also, they were sampled in the same area during a study in 2006 but this was unreported (David Conway *personal communication*). In this present study, they were sampled only by CDC-LT in Sare Seedy and Ngedden in the middle of the country, in an area that was predominantly occupied by *An. coluzzii* (**Table 5**, **Figure 10**).

Table 5: Species composition estimated from two sampling methods; CDC-LT and those collected from larvae or house searches in 2013 and 2014. For those sampled by CDC-LT, they represent blood-fed mosquitoes sampled in 2013.

				n (%)						
Collection Method	year	insecticide	N	*An. gambiae s.s.*	*An. coluzzii*	*An. arabiensis*	*An. gam x An. col hybrid*	*An. melas*	*An. funestus*	others
Larvae/house searches	2013	DDT	493	138	216	129	10			
		Deltamethrin	486	167	208	101	10			
		Total	**979**	305(31.2)	424(43.3)	230(23.5)	20(2)			
	2014	DDT	479	136	63	277	3			
		Deltamethrin	483	113	193	175	2			
		Total	**962**	249(26)	256(26.6)	452(47)	5(0.5)			
CDC-LT	2013		**714**	263(23)	82(11.4)	93(13)	3(0.4)	46(6.4)	326(46)	1(0.1)

House searches samples refer to offspring got from gravid mosquitoes collected inside houses. Missing values are NA, where no species were sampled. Only 94 blood fed mosquitoes were sampled in 2014 by CDC-LT and were excluded from analysis. House searches data in 2013 is the same data reported in chapter 2.

Larvae and day time house searches for blood-fed mosquitoes sampled two hybrids of *An. arabiensis* and *An. coluzzii*, one hybrid between *An. arabiensis* and *An. gambiae* s.s., and one *An. melas.* Species composition and proportion varied between villages and year (**Figure 11**) although there was a considerable degree of similarity in the number of shared species between village pairs within each year (Table 6, **Appendix 9**). Comparison of species distribution between CDC-LT and larval collection was not done here because CDC-LT catches represent only blood-fed mosquitoes and not representative of all species.

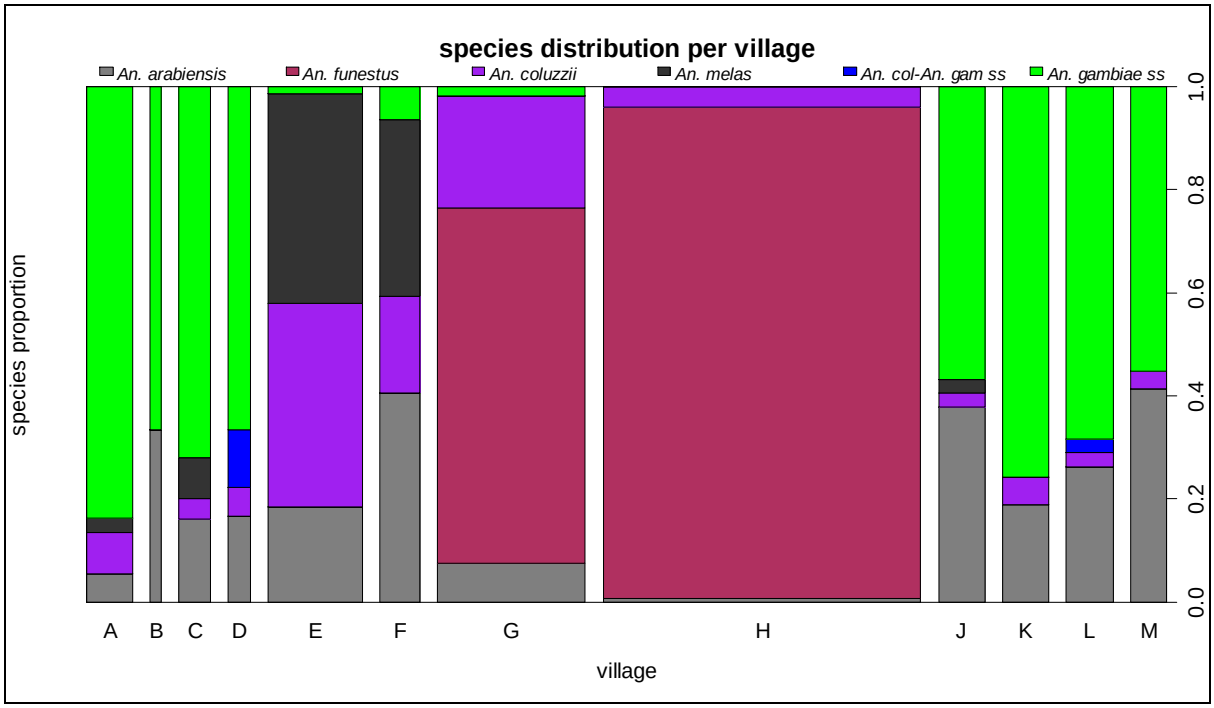

Figure 10: Species distribution of blood-fed anophelines collected using CDC-LT in the transmission season of 2013 in The Gambia. Width of bars denote sampe size (see **Appendix 10**). Letters denote village names; A- Bessi, B-Ndemban Tenda, C-Chogen Wellingara, D-Yallal Ba, E-Sinchu Njengudi, F-Dongoro Ba, G-Sare Seedy, H-Ngedden, K-Madina Samako, J-Njaiyel, L-Sare Wuro, M-Gunjur Koto.

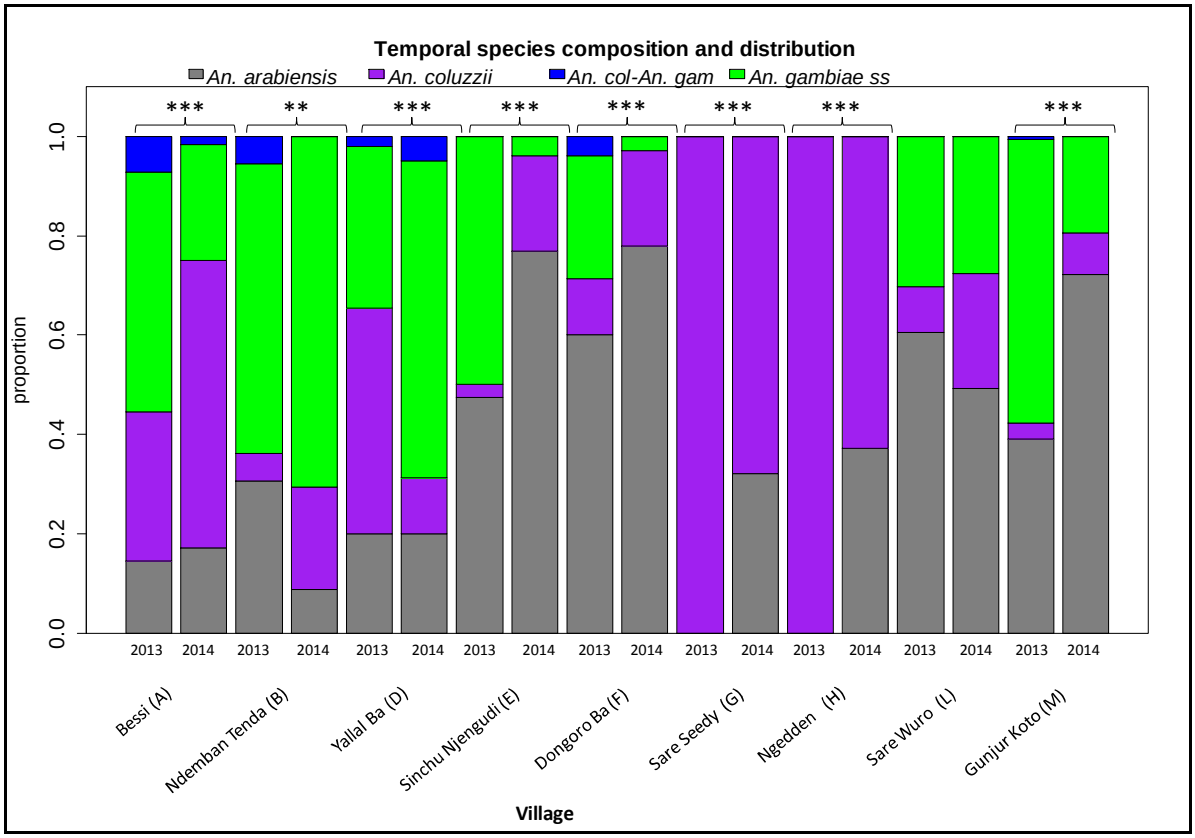

Figure 11: Species composition and proportion of malaria vectors sampled from larvae in the transmission seasons of 2013 and 2014 across The Gambia. Asterisks denote significant differences in species diversity per year assessed using Chi-squared tests; *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$. *An col-An gam* refer to hybrid between *An. coluzzii* and *An. gambiae s.s.* See **Appendix 11** for actual data per village.

Table 6: Similarity in species composition of F0 and F1 *Anopheles gambiae* s.l. as sampled by larval collections and house searches for blood-fed mosquitoes between high and low transmission villages during the transmission seasons of 2013 and 2014.

		Yue and Clayton index, θ		Euclidean distance, D,	
Region	Village pair	2013	2014	2013	2014
Region 1	Bessi and Ndemban Tenda	0.8	0.5	0.3	0.6
Region 2	Chogen Wellingara and Yallal Ba	NA	0.7	NA	0.4
Region 3	Sinchu njengundi and Dongoro Ba	0.8	1	0.3	0
Region 4	Ngedden and Sare Seedy	1	1	0	0.1
Region 6	Sare Wuro and Gunjur Koto	0.8	0.9	0.4	0.2

Both indexes have been calculated based on species proportions. For Yue and Clayton index, θ, 0 imply dissimilarity, 1-similarity. For Euclidean distance, D, 0 imply similarity, 1-dissimilarity.

3.3.1 *An. gambiae* s.l. phenotypic resistance to DDT and deltamethrin

DDT and deltamethrin susceptibility of *An. gambiae* s.l. varied between the two years, with susceptibility slightly increasing in some villages in 2014 (**Figure 12**). Variation in phenotypic resistance was significantly explained by the fixed terms; *Vgsc-1014 (F, S),* region, species and year except *GSTe2-114T* and *Vgsc -1575Y*. Although GSTe2 and N1575Y appeared not to contribute significantly to the model fit, the best model was the one that included all these as predictors of survival, suggesting their additional roles in conferring phenotypic resistance to DDT (**Table 7**).

Analysis of GLMM output indicated a general reduction in survival against the two insecticides in 2014 probably due to increased susceptibility in *An. gambiae* s.s to DDT in Sinchu Njengudi (E), Dongoro Ba (F) and Gunjur koto (M) and to deltamethrin in Sare Wuro (L). All possible genotype combinations of resistance mutations at the *Vgsc-1014* gene locus; *FF, LF, LS, SS* conferred survival advantage against DDT relative to the wild type (**Appendix 12**).

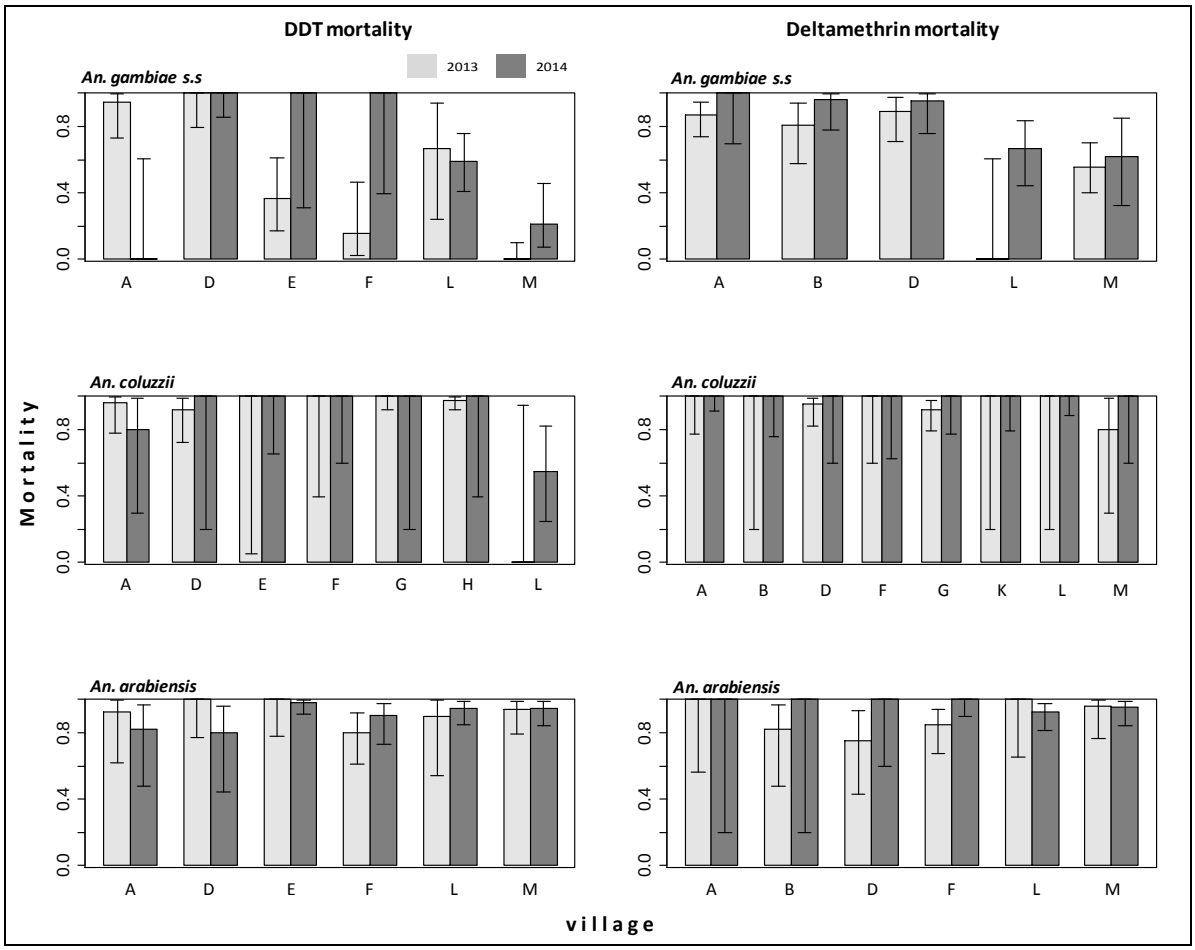

Figure 12: Twenty-four hour post exposure mortality estimates for malaria vectors sampled in the transmission seasons of 2013 and 2014 to DDT and deltamethrin as tested by WHO tube test bioassay. Only villages with data for two years are presented.

Table 7: Generalized Linear Mixed Effect Model testing the effects of *Vgsc-1014* (*F/S*), region, species and year and village as a random effect on 24 hour post exposure bioassay mortality to DDT.

Fixed terms	Chisq	Df	P
Vgsc-1014 (F,S)	72.21	5	<0.001
Region	30.39	5	<0.001
Species	8	2	<0.05
Year	8.29	1	<0.01
Gste2 – 114T	3.34	2	0.188
Vgsc - N1575Y	3.52	2	0.172

For deltamethrin, due to the small number of survivors in other species, analysis of bioassay mortality using a mixed effect model was only performed for *An. gambiae* s.s (**Table 8**). Only *Vgsc*-1014F and year significantly explained variation in the data (**Appendix 13**). Villages that had zero mortalities or complete susceptibility to deltamethrin were excluded to avoid boundary effect in the model (Yusuf et al., 2014, Williamson et al., 2013). The *Vgsc*-1014F homozygous mutants had survival advantage, relative to the wild type, while heterozygous at this locus had a non-significant survival advantage (**Table 8**).

Table 8: Generalized Linear Mixed Effect Model testing the effects of *Vgsc*-1014F, region and year and village as a random effect on bioassay mortality to deltamethrin. Estimates are log odds (logit) of *An. gambiae s.s* surviving deltamethrin

	Estimate	Std. Error	z value	p	
(Intercept)	-2.15	0.37	-5.79	<0.001	***
1014 FF	1.98	0.69	2.84	<0.01	**
1014 LF	1.03	0.55	1.89	0.058	.
region2	-0.33	0.6	-0.55	0.583	
region3	0.35	0.8	0.44	0.661	
region6	0.42	0.71	0.59	0.554	
year2	-0.86	0.38	-2.26	<0.05	*

Asterisks denote factor levels that significantly explained variation in mortality; *** p< 0.001, ** p<0.01 and * p< 0.05.

3.3.2 Insecticide resistance and malaria prevalence

There was no difference in insecticide susceptibility between high and low prevalence villages although malaria prevalence rates were significantly lower in 2014 probably due to the mass drug administration coupled with little precipitation. Consequently, there was no significant difference in malaria prevalence between high and low malaria prevalence villages (**Appendix 14**).

For villages initially classified as having high malaria prevalence, the number of *An. gambiae* s.s. surviving deltamethrin increased as one moved from western to eastern villages both in 2013, χ^2 =35, p < 0.001 and 2014, χ^2 =9, p < 0.01. For DDT, this trend was only observed in 2013, χ^2 =16, p < 0.001 and not in 2014 probably due to reduced resistance observed in this year. Similar trends were observed for low malaria prevalence villages for DDT in both years, 2013 χ^2 =60, p < 0.001 and 2014, χ^2 =41, p < 0.001 and deltamethrin 2013, χ^2 =6, p = 0.01 and 2014, χ^2 =8, p < 0.01.

In both years, the frequency of *Vgsc-L1014F* among *An. gambiae s.s.* sampled in the east was higher than that in western populations. Conversely, the *Vgsc-L1014F* frequency in western *An. gambiae s.s.* was less than 15 %. Coincident with increased susceptibility to insecticides, the frequency of this mutation in Central Gambia decreased from 61% to 21 % but the small sample size in 2014 led to highly uncertain estimation. The metabolic resistance marker, *Gste2-114T,* in *An. gambiae s.s.* was not widespread, with the highest frequency only at 20% in region 2 in the second year.

Despite no history of carbamates or organophosphate use in The Gambia until late 2014, the frequency of *Ace-1 119S,* ncreased, from 1% in 2013 to 7% in 2014 in region 6 (χ^2 =11.6, p = 0.001). For A*n. coluzzii,* the frequency of *Gste2-114T,* is approaching fixation in most regions whilst the *Vgsc*-1014F remains at a low frequency, at about 2%. *An. arabiensis* mainly carried the Vgsc-1014S mutation with a frequency of about 30% while Vgsc-1014F mutation stood at about 2% (**Appendix 15**).

3.3.3 Blood meal choice of malaria vectors

To identify the host choice for blood meal, all blood fed *An. gambiae* s.l. and *An. funestus*, that were sampled were screened. The source of blood meal could be identified in 49% (348) of all anophelines screened. Vertebrate hosts included humans (47%), cows (34%), dogs (0.9%), goats (7%), donkeys (7%) and a mixed blood meal of human and goat (2.6%), human and cow (0.3%), cow and dog (0.3%) and cow and goat (0.9%) (**Figure 13**, **Appendix 16**).

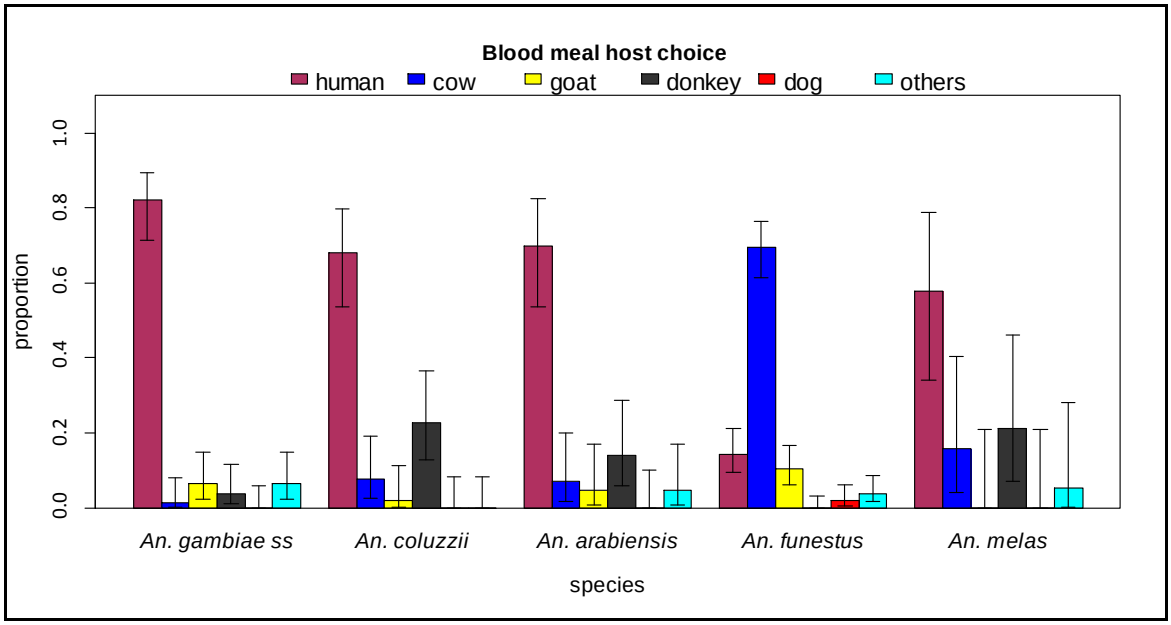

Figure 13: Sources of blood meals for malaria vectors collected using CDC-LT expressed as proportions with 95% binomial confidence intervals around the proportions. The data represent collections made during the transmission season of 2013. Sample size is detailed in **Appendix 16**.

All species except *An. funestus* fed on humans but the proportions of vertebrate hosts differed among species, $\chi^2 = 213$, $p < 0.001$ (**Figure 14**, **Figure 15**; see **Appendix 17** for actual data). There was no trend for increased feeding on humans moving from west to east of the country for all species nor difference in anthropophilic rate (proportion of human blood meals) between high and low prevalence villages (**Appendix 18**). *An. funestus* fed mainly on cows than any other vertebrate, $\chi^2 = 26.5$, $p < 0.001$. No difference in blood meal choice was detected for *An. gambiae s.s.* with any of the insecticide resistance markers screened (**Appendix 19**).

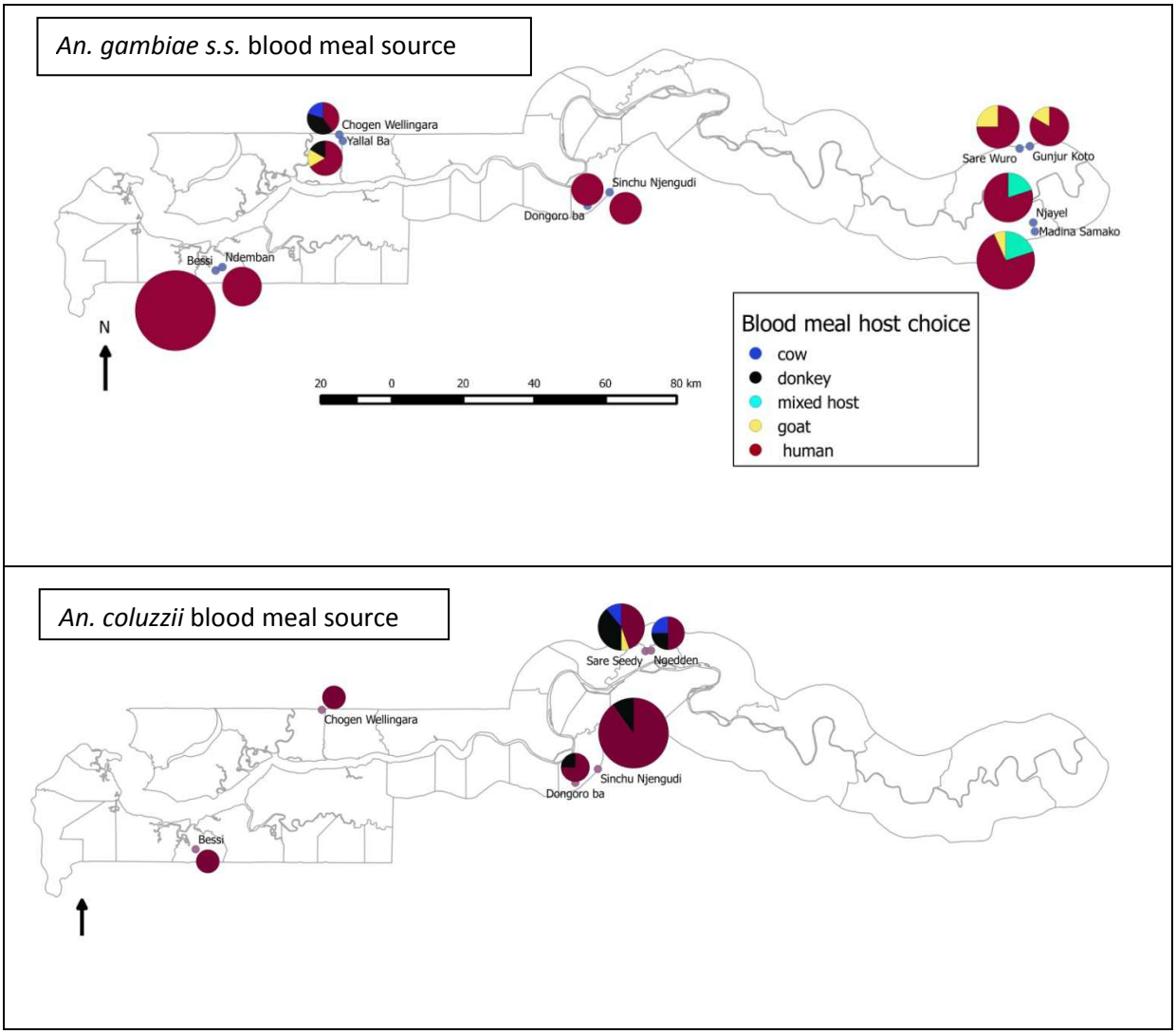

Figure 14: Blood meal host choice for *An. gambiae ss* (top) and *An. coluzzii* (below) samples collected using CDC-LT in the transmission seasons of 2013 across the Gambia. The size of the pie chart represents sample size (See **Appendix 17**).

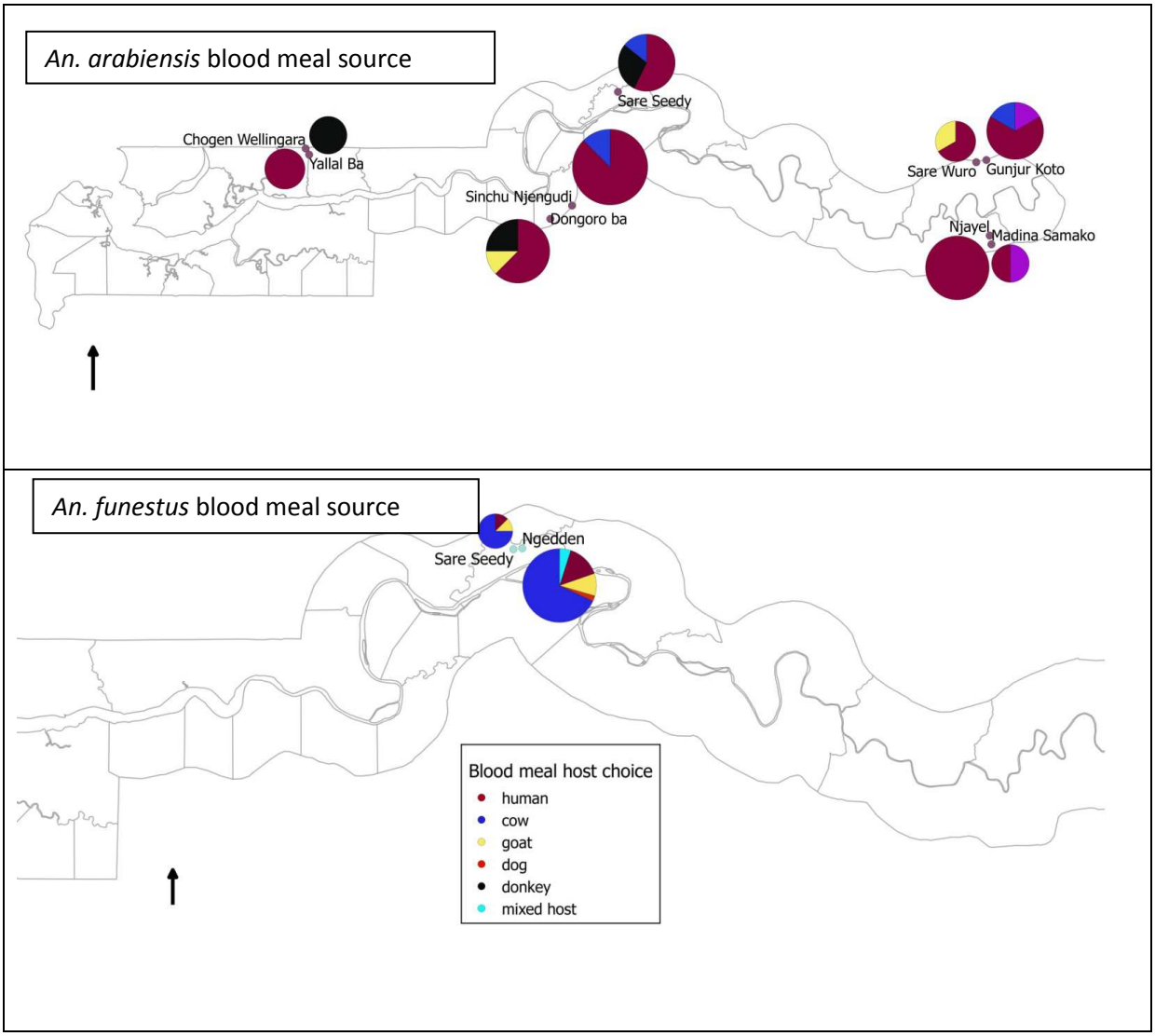

Figure 15: Blood meal host choice for *An arabiensis* (top) and *An. funestus* (bottom) samples collected using CDC-LT in the transmission seasons of 2013 across the Gambia. The size of the pie chart represents sample size (See **Appendix 17**).

3.4 Discussion

In the two years, despite variability in susceptibility to insecticides among the villages, geographical patterns of resistance to DDT and deltamethrin remained the same with *An. gambiae s.s*. from east being more resistant than those from western parts of the country. The *Vgsc*-1014F/*S* was the major driver of resistance to DDT and deltamethrin. In region two, the frequency of resistance mutations increased in *An. gambiae s.s.* except *Gste2-114T* and *Ace-1-119S.* The frequency of *Ace-1-119S* mutation in eastern *An. gambiae s.s* rose from 1 to 7% between 2013 and 2014. While *Anopheles gambiae* s.l preferred to feed on humans relative to other hosts, *An. funestus* that has been reported for the first time in The Gambia preferentially fed on cows in the two villages that they were sampled from.

The species composition between high and low prevalence villages in both years varied for each pair of villages excluding Ngedden and Sare Seedy that only had *An. coluzzii*. Even so, in the second year these two villages only had two populations, *An. arabiensis* and *An. coluzzii* at almost equal proportions. However, assessment of the species similarity using ecological distance matrices showed near similarity in species composition, at least to those sampled from breeding habitats. This means that similar species were present in the study villages and the difference was only in their proportions. If different species contribute differentially to malaria transmission then it could be expected that species present will impact on malaria transmission.

The indices assess how similar the communities are based on proportions of similar species and significant difference may be observed in scenarios where the proportions are extremely different with near absenteeism in one community (Yue and Clayton, 2005). Their distribution in space therefore will depend on the underlying ecological conditions of the

region (Caputo, 2008). Species diversity and composition of adult anophelines as sampled by CDC-LT will be reported elsewhere (Jawara *et al* in preparation).

The *Vgsc*-1014F mutation's ability to strongly predict insecticide resistance especially in *An. gambiae s.s.* makes it an invaluable marker of resistance in these settings and monitoring the frequency of the allele can help in management of spread of insecticide resistance. However, in future when different insecticides are used for vector control, its strong link may wane and therefore screening for it and combining with bioassays will be useful. Increased frequency of this mutation in region two is a cause for concern for management of insecticide resistance in the country.

However, this might not be surprising considering that an earlier study conducted few kilometres away from the villages in region two, Chogen Wellingara and Yallal Ba identified resistant populations (Betson et al., 2009). The failure of this study to fully capture the resistance status could have arisen due to the inadequacy to sample enough mosquitoes in the few natural breeding sites that were present. This was further worsened when, in the second year of collection, little rainfall was experienced all over the country. An attempt to use artificial breeding habitats to lure gravid mosquitoes to lay eggs only got few extra samples with the majority being *An. arabiensis*, in which phenotypic resistance has not been detected as yet.

To the North of region two, is an area in Senegal where resistance has continually been reported in *An. arabiensis* and linked to *Vgsc-1014S* mutation (Ndiath, 2014). In this present study, the frequency of *Vgsc-1014S* in *An. arabiensis* stood at approximately 30% but linking this to phenotypic resistance was not possible due to fewer survivors to allow for association tests. Nonetheless, such high frequency with no link might suggest other resistance mechanisms involved in *An. arabiensis* in The Gambia.

Similarly, in the east, a previous study (Pinder et al., 2014) did not detect phenotypic resistance in samples tested, which were mainly *An. arabiensis*. More recently, in eastern Senegal, similar resistance patterns across malaria vectors as observed in this study have been reported (Niang et al., 2016) although they did not screen for all known resistance mutations as done presently. This *Vgsc-1014S* mutation however, has been liked to resistance in *An. gambiae* s.s. and *An. arabiensis* other areas of SSA (Mawejje et al., 2013, Kabula et al., 2014).

Changes in weather patterns could impact on disease system. Little rain in 2014 may have led to fewer breeding sites for mosquitoes. Consequently, lower parasite rates were recorded in that year. This link however, is confounded by mass drug administration with DiHydro Artemisinin Peperaquine which took place before the start of the transmission season and may have cleared majority of parasites. The heterogeneous nature of insecticide resistance both spatially and temporally precludes studies that attempt to investigate the possible roles that insecticide resistance plays in malaria transmission especially if studied within a short time scale like herein. It is crucial that resistance status be monitored over a period of time to be able to document stable patterns of transmission with insecticide resistance as has been demonstrated at one site in Senegal (Trape et al., 2011).

In addition to *Vgsc*-1014F mutation that is now common and spreading in The Gambia, the increase in frequency of *Ace-1-119S* poses operational challenges for vector control that now uses bendiocarb for IRS. The driver of this resistance allele is not established in this setting although the allele has been demonstrated in samples from Vellingara, approximately 70 kilometres eastward inside Senegal (SNMCP, 2011). Also, though not documented, the application of pesticides of similar mechanisms of action to organophosphates/carbamates in agricultural farmlands may also contribute to this.

Whether the malaria vectors inside Gambia mix with resistant inland populations from Senegal remains to be established. Since the GNMCP made an operational switch from DDT to bendiocarb for IRS activities beginning 2015, in line with WHO insecticide resistance management plan (WHO, 2012), dynamics of *Ace-1*-119S is worth monitoring.

Although sample size limits interpretation of blood feeding behaviour, the choice for blood meal host for malaria vectors in The Gambia remain to be human except *An. funestus* that preferred to feed on cows. Even in villages that were considered of low malaria prevalence, malaria vectors preferred the human host.

The appearance of *An. funestus* in The Gambia and its preference to feed on cow indicates that it may not be involved in local malaria transmission although despite its wide choice of blood meal host, it has been implicated in malaria transmission elsewhere where it is the major malaria vector (Lochouarn et al., 1998, Dia et al., 2003, Vezenegho et al., 2013).

It is probable that this species may have migrated from Senegal that has perennially had *An. funestus.* A study conducted in Senegal along the Gambian border to the north showed *An. funestus* feeding on multiple hosts but was greatly implicated in local transmission of malaria (Lochouarn et al., 1998). Whether ecological differences between Senegal and Gambia hindered this species from habiting Gambia remains to be established and also if the ecological conditions changed in region 4 of the study sites.

Generally, the difficulty in sampling *An. funestus* from natural breeding habitats and breeding them in the laboratory is always challenging and could help explain why they were not sampled in larval habitats or in house searches for blood-fed samples but only in CDC-LT and human landing catches (will be reported elsewhere, Jawara *et al*).

Sample size limited the investigation of whether anthropophilic rates differ between resistant and susceptible populations. Behaviourally resistant mosquitoes may be diverted to feed on other animals but there was no evidence either way to support diversion or not. This diversion however, would hold where humans completely utilize the LLINs but if humans are exposed to foraging mosquitoes while still indoors, they would still be bitten (Seyoum et al., 2012). Depending on host availability, malaria vectors (resistant or susceptible) will bite available hosts in their quest for vertebrate blood meal (Killeen et al., 2001).

Conclusion

The broad pattern of geographical differences in insecticide resistance to DDT and deltamethrin in *An. gambiae* s.s. remained stable. While western villages had less resistance compared to eastern villages, the increase in *Vgsc*-1014F frequency indicates resistance could be spreading or *de novo* mutations could have arisen in the western populations. *Anopheles funestus*, reported in The Gambia for the first time, preferred feeding on cow compared to *Anopheles gambiae* s.l that preferred humans as a source of blood meal. The stability of insecticide resistance to DDT and deltamethrin especially in eastern Gambia gives credit to the Gambian National Malaria Control Programmes for the operational switch to bendiocarb for IRS activities. However, a cause for concern is the spreading of *Ace-1-119S* mutation following scale –up of bendiocarb use in IRS activities that will potentially limit malaria control achievements.

4 CHAPTER 4: IMPACT OF AGE ON SUSCEPTIBILITY OF RESISTANT MALARIA VECTORS TO DDT IN THE WILD

Impact of age on susceptibility of resistant malaria vectors to DDT in the wild.

Abstract

Background

Insecticide resistance which increases adult vector longevity may increase the probability of malaria transmission as a larger proportion of individuals may survive the extrinsic incubation period. Previous studies have shown that older mosquitoes become more susceptible to insecticide with age, but this has not been studied in wild caught adults. This preliminary study investigated the relationship between age and insecticide resistance in wild *Anopheles gambiae* populations.

Methods

Adult mosquitoes were collected from five villages with varying resistance levels to DDT. Females were tested for phenotypic resistance using WHO tube exposures to DDT and bendiocarb; parity status was determined as a proxy for age. All were screened for known molecular markers of resistance to DDT and bendiocarb and also infection with *Plasmodium*.

Results

Of 265 mosquitoes sampled and tested for resistance, 251 (167 exposed to DDT, 84 to bendiocarb) were successfully identified as *Anopheles gambiae* s.s (69.3%), *An. coluzzii* (7.6%) and *An. arabiensis* (23.1%). There was no difference in DDT and bendiocarb mortality between nulliparous and parous mosquitoes $\chi^2_{df=1} = 0$, p = 1. The proportion of parous *An. gambiae* s.s. that carried the *Vgsc*-1014F allele was higher than for those nulliparous ($\chi^2_{df=1} = 5.7$, p = 0.02). Irrespective of parity status, target site mutations

conferred a strong survival advantage, to DDT from *Vgsc*-1014F (OR 18, 95% CI 2-557, p < 0.01), and to bendiocarb from *Ace-1*-119S (OR 41, 95% CI 4-1394, p < 0.01).

Conclusion

Presence of *Vgsc*-1014F predicted resistance phenotype in mixed age populations of *An. gambiae s.s* and upon exposure, older mosquitoes still withstood the effect of insecticides that they were resistant to when young. The higher frequency of *Vgsc*-1014F in older mosquitoes compared to younger ones suggest that mosquitoes undergo selection in the wild and therefore have survived insecticide exposure better and could explain the difference from laboratory results that have used mosquitoes raised without selection. The use of mixed physiological age populations may still be appropriate for monitoring insecticide resistance as recommended by World Health Organization. Where it is costly or logistically challenging to obtain sufficient samples for bioassays, established molecular markers may serve as a useful proxy for insecticide resistance status and may also be expected to predict a shifted age structure of local malaria vectors subject to application of insecticides to which they show resistance.

4.1 Introduction

In malaria transmission, older anophelines that have laid eggs (parous) are considered to be crucial epidemiologically because they will have had the opportunity to acquire *Plasmodium* parasites from bites on an infected human host (Gillies and Wilkes, 1965). In sub-Saharan Africa (SSA), proportions of parous mosquitoes rise toward the end of rainy seasons coinciding with increasing prevalence of people harbouring malaria parasites (Obala et al., 2012, Thomson et al., 1994) outlining the important link between vector longevity and malaria transmission. Theoretical studies of malaria transmission showed how transmission potential of malaria vectors was most affected by vector longevity (Macdonald, 1956). Hence, to control malaria, reducing the survival rates of adults to reduce the numbers of older, infective mosquitoes becomes paramount. Present malaria control strategies that target adult anophelines using Long Lasting Insecticidal bed Nets (LLINs) and Indoor Residual Spraying (IRS) attempt to achieve this by killing the malaria vectors before they grow old enough to transmit the parasite (WHO, 2015a).

Following widespread deployment of these control methods, malaria vectors over much of Sub-Saharan Africa (SSA) have developed resistance against the cheap insecticides, pyrethroids and DDT, commonly used in the LLINs and IRS activities (Ranson et al., 2011). As reviewed in chapters one and two, this creates challenges to malaria control programmes (Hargreaves et al., 2000, Trape et al., 2011) although demonstrating a direct impact of resistance remains difficult (Kleinschmidt et al., 2015).

Understanding the roles that insecticide resistance plays in malaria transmission requires insight into how adult vector longevity is affected. Although studies investigating this relationship have shown increasing susceptibility to insecticides with age (Chouaibou et al., 2012) (Rajatileka et al., 2011) (Jones et al., 2012b), they are limited in interpretation due to

the longer holding time in the laboratory which can influence phenotypic traits. Furthermore, other general changes in the genome that arise due to laboratory environment might contribute to reduced overall fitness of populations and thus affect phenotypic traits (Huho et al., 2007) (Kassen, 2002). In another study in Tanzania (Lines and Nassor, 1991), wild caught mixed-age specimens tested directly showed lower resistance compared to newly emerged adults from wild caught larvae. However, when the adults that emerged from larvae were kept for longer in the laboratory, they readily died and resistance was significantly much lower compared to mixed aged adults tested directly from the wild. Since the age of the wild caught adults was undetermined, the influence of age is not discernible.

A challenge of using wild caught mixed ages is a lack of standardization in physiological states that may reduce comparability between sampling locations and/or temporal samples from the same location. Physiological states such as ingestion of blood by insect vectors typically trigger elevated expression of many genes (Marinotti et al., 2006) and may also affect insecticide resistance.

Among the proteins over-expressed in blood feeding are members of detoxification enzyme families (esterases, glutathione-S-transferases and P450s) (Marinotti et al., 2006) which are frequently implicated in conferring resistance or supplementing other resistance mechanisms (Mitchell et al., 2014, Riveron et al., Riveron et al.). Indeed, resistant laboratory strains, blood fed multiple times after every three days has been shown to remain more resistant with age compared to their unfed counterparts (Oliver and Brooke, 2014).

Continual exposure to environmental stressors, coupled with variation in age of mosquitoes and their physiological state makes prediction of insecticide resistance in unstandardized wild samples uncertain. As a consequence, predicting the phenotypic resistance status of both young and old mosquitoes using genetic markers may not be straightforward.

Genetic markers might be applicable to wild caught mixed aged adults tested directly from the field because they are continually exposed to insecticides. If resistant populations are heavily reliant on target site resistance for survival to DDT and bendiocarb, selection for the target site resistance mechanisms could be selected. Thus, resistance may decline less than predicted with age from laboratory experiments and the frequency of resistance allele would be predicted to be higher in older adults.

Alternatively the same pressure in the wild could lead to induction, rather than selection, for resistance via elevated gene expression. Whilst again this could lead to higher resistance in older females than expected from laboratory studies, the effect could be predicted to be transient and reduce following housing in benign laboratory holding conditions. However, it is not known whether target site resistance marker would be predictive of phenotypic status in wild caught mixed aged mosquitoes.

In the Gambia, *Vgsc*-1014F causes strong resistance to DDT and pyrethroids in *An. gambiae* s.s (chapter two;(Opondo et al., 2016)). It is important to note that exposure of mosquitoes to insecticides in the wild is not uniform (Molineaux et al., 1979) and therefore weaker individuals may still be alive because they are not exposed. Nevertheless if exposure is sufficient to provide selection, genetic marker can be applicable to both nulliparous and parous mosquitoes.

Pragmatically, it might be best to use the markers instead of phenotyping especially in settings where high resistance allele frequency in the population make it challenging to do genotype-phenotype association tests because of the high sample size needed to see significant association (Donnelly et al., 2009, Gordon et al., 2002). This may even be more useful in settings with generally lower vector densities like The Gambia where sampling enough specimens to perform bioassays may be logistically challenging for the otherwise

poorly resourced National Malaria Control Programmes (NMCPs). Therefore, screening for known molecular markers of resistance to infer resistance can provide a useful proxy (Donnelly et al., 2015, Weetman and Donnelly, 2015).

Thus, this preliminary study attempted to investigate whether older *An. gambiae s.s* mosquitoes caught and exposed to DDT and bendiocarb would survive as well as their younger counterparts in the wild and to investigate the predictability of resistance in different age classes using genetic markers.

4.2 Methods

4.2.1 Study design

The study was carried out in five villages, selected from 12 villages that are part of a study investigating malaria transmission dynamics (Chapter two study villages; Mwesigwa *et al* in preparation), with varying insecticide resistance status and pressures. Three eastern villages, Gunjur koto (M), Sare Wuro (L) and Sinchu Njengudi (E) were earlier identified as having *An. gambiae* s.s. that were both resistant and susceptible to DDT and pyrethroids while two villages, Ndemban Tenda (B) and Ngedden (H) had only susceptible malaria vectors (Chapter two; (Opondo et al., 2016)). In each village, adult mosquitoes were collected by human landing catches (HLC) (Service, 1977) performed by eight trained volunteers sitting one to two metres from houses. Sampling took place from 1900 – 0600 in the months of September and October 2013.

Outdoor HLC was chosen because indoor catches had not yielded many mosquitos in the same period during routine collections (Musa Jawara *personal communication*), and it was the only outdoor sampling method that would keep specimens alive and in good condition. Sampled mosquitoes were then transported in an air conditioned car the following morning to the MRC field station, Wali Kunda where they were held in the insectary for 2 days and provided with 10% glucose *ad libitum*. The two days allowed those fatigued and physically hurt specimens to be separated and to allow for stabilization of conditions in the insectary. Those fatigued would be expected to be immobile on the floor of the cage or dead. All adult mosquitoes were reared under the same environmental conditions within the insectary alongside the susceptible *An. coluzzii* Yaoundé strain maintained in the laboratory.

On the third day, the sampled adult female mosquitoes were exposed to WHO tube test bioassays for resistance phenotyping against DDT and bendiocarb. Random samples of

different ages drawn from the Yaoundé strain, acting as control, were also exposed to DDT and bendiocarb. After insecticide exposure, the mosquitoes were transferred to holding tubes and fed on 10% glucose.

Mortality 24 hours post-exposure was recorded for all the specimens. Immediately upon recording mortality status, starting with the dead mosquitoes, the mosquitoes were dissected to reveal the ovary and parity status determined according to pattern of tracheole skeins (Detinova, 1962). Within the ovary, presence of coiled tracheole skeins indicate nulliparous while uncoiled tracheole skeins indicate parous mosquitoes.

The head, thorax and legs of each mosquito were stored in individual Eppendorf tubes with silica gels and transported to MRC Fajara laboratories for molecular diagnostics. DNA from all mosquitoes was extracted using the DNeasy® Blood and Tissue kit according to manufacturer's protocol (Qiagen). Scott's protocol (Scott et al., 1993) was used to identify *An. gambiae s.s, An. arabiensis, An. melas* while the SINE-PCR (Santolamazza et al., 2008b) protocol was used to further distinguish among *An. gambiae s.s, An. coluzzii* and *An. arabiensis*.

All mosquitoes tested in the insecticide resistance bioassay were genotyped for *Vgsc*-1014F, *Vgsc-1575Y, Gste2*-114T and *Ace1*-119S using Taqman assays (Bass et al., 2007b, Bass et al., 2010, Jones et al., 2012a, Mitchell et al., 2012). *Plasmodium* infection status was also determined using a Taqman protocol (Bass et al., 2008).

4.2.2 Statistical analysis

All statistical analyses were performed in the R statistical package (R version 3.1.2, 2014-10-31). Mortality in the control group was always less than 5% so no mortality correction was

applied. Odds ratios and Fisher's exact tests were used to measure the association between age and susceptibility to insecticides. Allelic odds ratios (Clarke et al., 2011) were used to measure association between resistance alleles and *Plasmodium* infection status. Proportionality tests (Newcombe, 1998a) were used to establish differences in mortality between groups. To assess the effect of reproductive age (herein referred to as age) on insecticidal mortality, *An. gambiae* samples were categorized into the three age groups, nulliparous, parous and gravid. Populations from east and west of the country were analyzed separately because of the stark contrast in susceptibility to DDT between *An. gambiae ss* from east and west of Gambia.

4.2.3 Ethical clearance

This study was approved by MRC scientific coordinating committee and ethical clearance obtained from The Gambia Government / MRC Joint Ethics committee. Informed oral consent was obtained during village sensitization meetings. Signed consent forms were filled by volunteers performing HLC after thorough explanation. Volunteers were given prophylaxis before start of HLC and upon completion of the HLC exercise, they were tested and those found to be positive were treated. Those performing the HLC were paid on a scale of a daily paid worker at MRCG.

4.3 Results and Discussion

A total of 265 female adult *An. gambiae* s.l. mosquitoes were collected from the five villages and assayed for phenotypic resistance to DDT and bendiocarb using WHO tube tests. Laboratory reared female adult mosquitoes, from the Yaoundé (Cameroon) colony, were used as controls. With the exception of 14 samples that failed to amplify, all field collected mosquitoes were identified to species using molecular methods. This yielded a total of 251 mosquitoes; of these, *Anopheles gambiae* s.s constituted 69.3%, *An. coluzzii* 7.6% and *An. arabiensis* 23.1% (**Figure 16**) of which parous females constituted approximately 65% (**Table 9**). From this total collection of *An. gambiae* s.l., 167 were exposed to DDT (89 alive, 78 dead), and 84 were exposed to bendiocarb (3 alive, 81 dead).

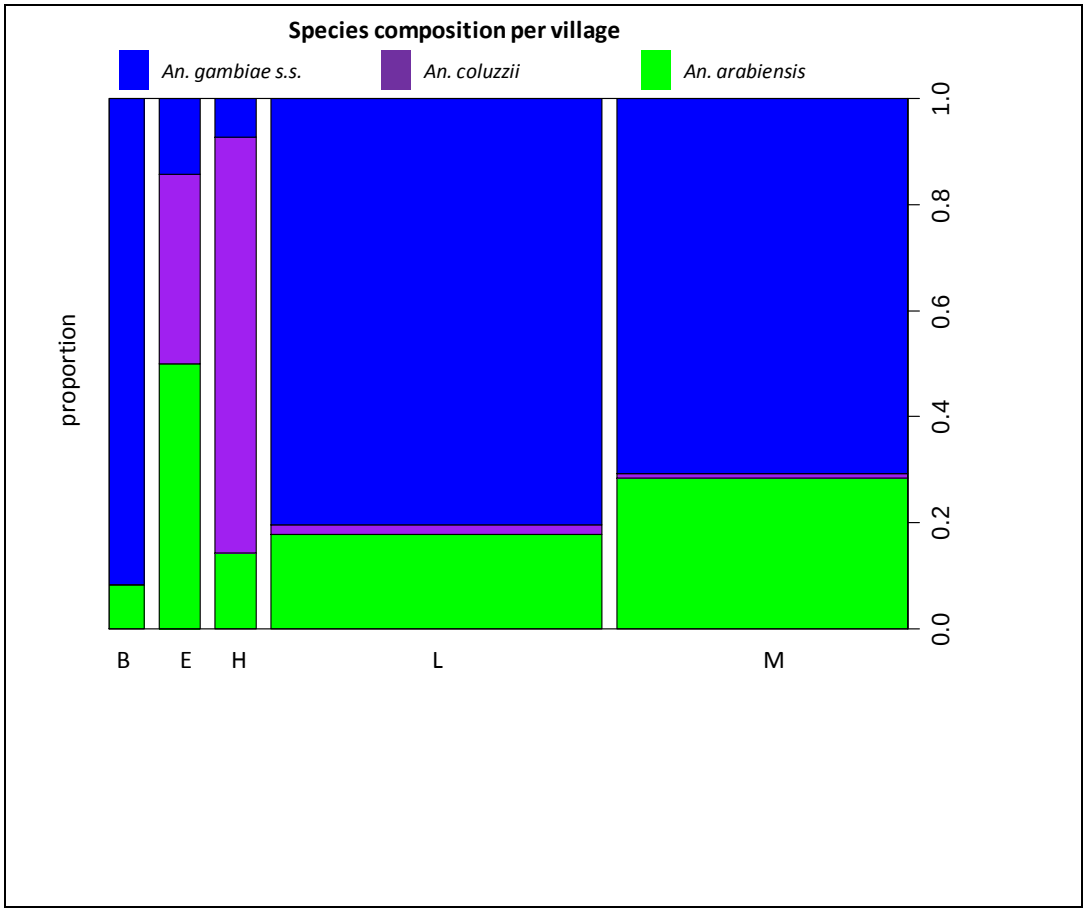

Figure 16: Species distribution and composition of mosquitoes collected by Human Landing Catch between October and November 2013 in five Gambian villages; B – Ndemban Tenda, E – Sinchu Njengudi, H – Ngedden, L – Sare Wuro and M – Gunjur Koto. Width of the bars represents sample size (see **Appendix 20)**.

This study further confirms that resistance to DDT in *An. gambiae* s.s. is prevalent even in wild caught adults from eastern Gambia. There was variation in susceptibility to DDT among species with *An. gambiae s.s* more resistant than the other two species, test of proportions $\chi^2_{df=2} = 65.3$, $p < 0.001$, (**Figure 17**). For bendiocarb however, there was no discernible

difference in susceptibility with all species exhibiting mortality levels in excess of 98 % (**Figure 17**). Paucity of other species limited the ability to conduct association tests between mutations and susceptibility to insecticides (**Appendix 21**) hence all the analyses focused on the most abundant species, *An. gambiae s.s*.

Table 9: Distribution of reproductive age assessed using parity rates of malaria vectors sampled using HLC in five villages in the transmission season of 2013

	Physiological age						
Species	**Nulliparous (nu)**	**Parous (p)**	**Gravid (g)**	Total (N)	**Parity rate, %, (p+g)/N**	**95 % Confidence Intervals of parity rate**	
						Lower	**Upper**
An. gambiae s.s	22	118	26	166	86.7	80.4	91.3
An. arabiensis	17	28	8	53	68	53.6	80
An. coluzzii	6	8	3	17	65	38.6	85

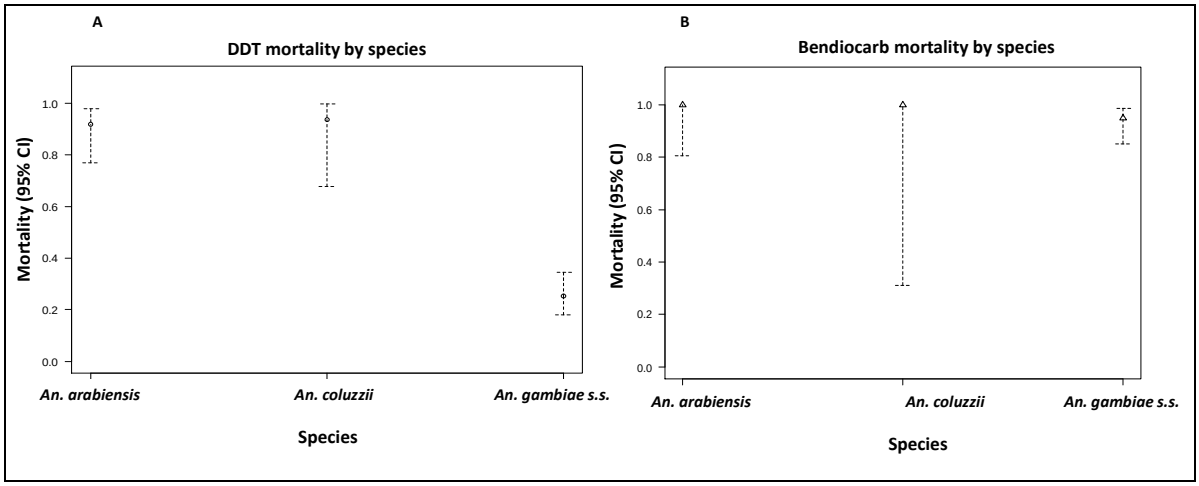

Figure 17: Twenty-four hour post-exposure mortality of field caught *An. gambiae* s.l to DDT (A) and bendiocarb (B) from the five collection villages (pooled).

In the east where resistance to DDT/pyrethroids is strongest, both nulliparous and parous adult populations survived DDT exposure with no difference in mortality levels, $\chi^2_{df=1} = 0$, p = 1. Resistance to bendiocarb is not widespread, and mortality was high in both nulliparous and parous populations (**Figure 18**) with no difference in mortality, $\chi^2_{df=1} = 0$, p = 1 suggesting that resistance to insecticides in the wild may not be age dependent. The major driver of insecticide resistance to DDT (and possibly pyrethroids) in *An. gambiae* s.s. in The Gambia is *Vgsc*-1014F, OR 18, 95% CI (2-557), p < 0.01.

For those samples tested for DDT, the proportion of parous mosquitoes carrying the resistance allele was higher than that in nulliparous samples ($\chi^2_{df=1} = 4.7$, p = 0.03) and this trend was the same even when tested on all samples collected in the east regardless of insecticide they were exposed to ($\chi^2_{df=1} = 5.7$, p = 0.02), odds ratio (OR 10.4, p=0.02). This suggests that older mosquitoes might be pre-selected following exposure to insecticides either on walls in the case for DDT or deltamethrin on LLINs or other similar insecticides used in

agriculture that could be transported into larval breeding grounds. This difference in resistance allele carriage is also consistent with the relative difference (albeit it not significant) in mortality between parous and nulliparous mosquitoes (**Figure 18**).

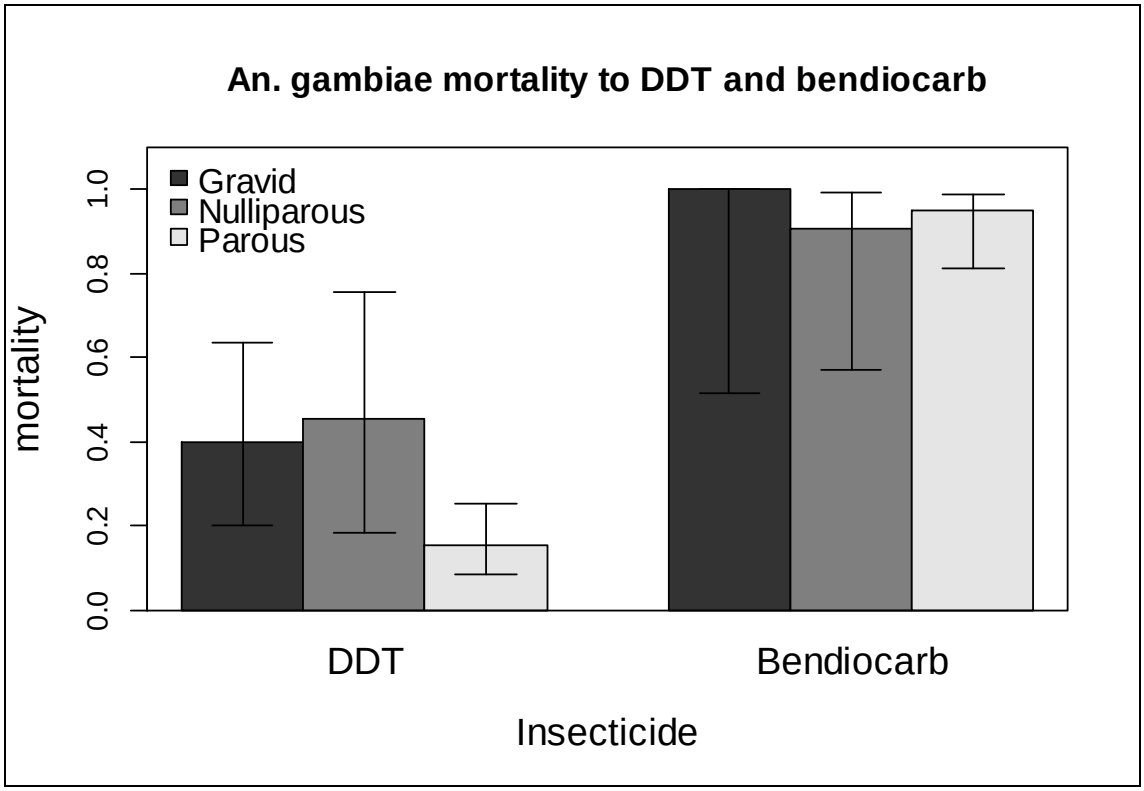

Figure 18: Mortality estimates with 95% confidence interval error bars of wild caught adult female *An. gambiae s.s* of different reproductive ages to DDT and bendiocarb.

If environment was inducing resistance, holding the mosquitoes in the laboratory for the two days would have possibly taken out 'environment induced resistance' meaning that this source of age-extended phenotypic resistance should have been nullified. However, since

paucity of mosquitoes did not allow for extended duration of holding time in the laboratory, this study cannot conclusively say how long the effect of 'environment induced' resistance lasts in wild caught adults kept in the laboratory. Although an earlier study (Lines and Nassor, 1991) conducted in Tanzania showed that newly emerged (chronologically younger) wild caught mosquitoes were significantly more resistant with about 95% surviving, the resistance of adults collected in the field was also relatively high with about 75% surviving DDT exposure.

The phenotypic resistance to DDT of 75% in wild caught adults though less than that seen in newly emerged adults, would still be enough to cause operational challenges and perhaps it represented the best estimate of the resistance status of populations and how they respond to control interventions in the wild. However, both the present and past studies are limited by the failure to truly establish the chronological or reproductive ages of wild caught females; beyond 1-parous for the present study and no age grading at all for the older study.

The influence of other molecular markers of resistance like *Gste2* (metabolic resistance marker) and *N1575Y* were not observed in this study but this could be influenced by small sample size reducing power of association tests of these markers with phenotypic resistance. However, in Chapter two (Opondo et al., 2016), these markers were also associated with resistance but their effect was masked by the strong presence of *Vgsc*-1014F. Of the samples carrying the *Vgsc*-1014F mutation, 97.5% (N=157) were *An. gambiae s.s* of which 90% were from the eastern villages. The remaining 2.5% (N=4) of *Vgsc*-1014F carriers were *An. arabiensis* while this mutation was not observed in *An. coluzzii* (**Table 10**).

Table 10: Distribution and allele frequency with 95% confidence intervals of molecular markers of resistance among malaria vectors collected by HLC.

Species	**N**	***Vgsc*-1014F**	***Vgsc-1575Y***	***Gste2*-114T**	***Ace-1*-119S**
An. gambiae s.s	174	0.89 (0.85 – 0.92)	0.2 (0.16 – 0.25)	0.12 (0.09 – 0.16)	0.06 (0.04 – 0.09)
An. arabiensis	58	0.04 (0.02 – 0.11)	0 (0 – 0.05)	0 (0 – 0.05)	0 (0 – 0.05)
An. coluzzii	19	0 (0 – 0.1)	0 (0 – 0.4)	0 (0 - 0.4)	0 (0 – 0.6)

N is the overall population sample but the denominator varies as some samples did not amplify.

Resistant *An. gambiae* s.s appears to be the most important malaria vectors in eastern Gambia although the involvement of other species, susceptible or resistant, cannot be ignored. Ten *An. gambiae* s.s. samples that tested positive for *P. falciparum* carried the carrying *Vgsc*-1014F allele, but there was a negative association observed between resistance mutation and infection status (**Table 11**), possibly due to sample size and the high *kdr* frequency. A total of 11 female *An. gambiae s.s* from Sare Wuro (n=6) and Gunjur Koto (n=5) tested positive for *Plasmodium falciparum.* One *An. arabiensis* from Sare Wuro was infected with either *Plasmodium ovale* or *P. vivax* or *P. malariae* read as *Povm* in the PCR output product (**Appendix 22**). Thus the sporozoite rate in Gunjur Koto was 5.1% while that in Sare Wuro, 5.3%.

This study hints at a higher transmission potential of resistant *An. gambiae* s.s. but this generality may not apply for *An. arabiensis* in this setting. However, recently in neighbouring Senegal, wild caught resistant *An. arabiensis* were more likely to be infected with *P. falciparum* oocysts and sporozoites than susceptible strains (Ndiath et al., 2014) and may have been involved in high malaria transmission in those settings (Ndiath, 2014). Similar

trends were observed in *An. gambiae* in Burkina Faso by Alout *et al* (Alout et al., 2013) but not Jones *et al* (Jones et al., 2012b). In Sri Lanka, resistance was thought to reduce the potential for filarial transmission (McCarroll and Hemingway, 2002) where the authors implied that fewer microfilariae in resistant populations was a proxy of reduced transmission potential.

Infection of mosquitoes with mainly *P. falciparum* indicates that it is the major malaria parasite in eastern Gambia despite detection of other *Plasmodium* species here and in previous studies (Thomson et al., 1994, Mwesigwa et al., 2015). Its role may be further indicated by the fact that they were sampled in the middle of the rainy season that has been associated with peaks of vector density (Lindsay et al., 1993a) and malaria transmission (Lindsay et al., 1989).

Table 11: Association between *Vgsc*-1014F and three phenotypes; mortality to DDT, infection status and parity in mixed ages of *An. gambiae* s.s collected by HLC.

			***Vgsc-L1014F* genotype**				
		N	Leu/Leu[#]	Phe/Leu	Phe/Phe[##]	Test statistic	P
DDT mortality	Alive	84	0	1	83	OR = 65*	0.000
	Dead	16	4	2	10	Exact [¥]	0.000
Infection status	*P. falciparum* positive	11	1	1	9	Exact [¥]	0.042
	P. falciparum negative	148	3	3	142	$\chi^2 = 3.8$[§]	0.05
Parity	Parous	118	6	2	110	Exact[¥]	0.000
	Nulliparous	22	5	1	16	$\chi^2 = 8.9$[§]	0.003
	Gravid	26	4	1	21		

[#] Leu (Leucine) is the wild type allele, [##] Phe (Phenylalanine) is the mutant allele. *****Odds ratio (OR) is the allelic odds ratio. [§] Test statistic and p-value from a χ^2 test of trend. [¥] Fishers exact test based on allelic frequency whenever one or more expected values was less than 5. For parity trend test, gravid mosquitoes were excluded as their reproductive age was not determinate.

The presence of *Vgsc*-1014F in *An. gambiae s.s.* may be used in place of the WHO tube test bioassay to infer resistance to DDT in The Gambia. The presence of *Vgsc*-1014F in mixed ages of wild caught anophelines and continued involvement in explaining mortality provides

support to the idea of screening for resistance mutations, where one has been established, to infer insecticide resistance especially where it is logistically challenging to collect enough samples for bioassays (Weetman and Donnelly, 2015, Donnelly et al., 2015).

An example of molecular marker shown to strongly predict resistance to organophosphates and carbamates in wild populations is *Ace-1*-119S (Essandoh et al., 2013, Edi et al., 2014b). In the present study, despite having few mutants at this locus, higher odds (OR 41, 95% CI (4-1393), $p < 0.01$) of surviving bendiocarb exposure were recorded. Their presence therefore is indicative of resistance both within populations and at an individual level.

In this study, the parity status was not established beyond 1-parous (those that have laid eggs once) and therefore not easy to correlate reproductive with chronological age which would allow correct assignment of resistance phenotype with age. Such studies may therefore benefit immensely from the new age-grading infrared technology (Sikulu et al., 2010) that allows for determination of chronological age to correlate with physiological age. Paucity of mosquitoes could not also allow for testing of effect of age on gene expression based resistance such as metabolic resistance.

4.3.1 Conclusion

There was no difference in survivorship between wild caught nulliparous and parous adult mosquitoes although studies that determine their chronological age explicitly such as use of infrared technology is advised to better inform vector competence studies. Selection of resistant populations to insecticides in the wild as they age appears to be important in maintaining phenotypic resistance in older mosquitoes, and seems a plausible explanation for the lack of decline of resistance with age commonly observed in laboratory studies.

The *Vgsc*-1014F is a strong marker of resistance to DDT (and possibly pyrethroids) in Gambian *An. gambiae s.s.* and its presence in both young and older mosquitoes makes it suitable as a proxy of resistance in absence of bioassay experiments. Both nulliparous and parous wild caught *An. gambiae* s.s. exhibited almost equal phenotypic resistance to DDT and bendiocarb and confirms WHO recommendation of utilizing wild caught mixed ages to assess levels of insecticide resistance. Irrespective of sample size, that all but one infected anophelines carried the *Vgsc*-1014F allele, indicates that in localities where there are resistant and susceptible populations, the roles of resistant populations in local malaria transmission should be investigated.

5 CHAPTER FIVE: POPULATION STRUCTURE AND ORIGIN OF INSECTICIDE RESISTANCE IN *ANOPHELES GAMBIAE* S.S. OF THE GAMBIA.

Population structure and origin of insecticide resistance in *Anopheles gambiae* s.s. of The Gambia

Abstract

Identification of barriers to gene flow between members of the same species is important for understanding the population structure and evolution of organisms and spread of important genes. The temporal persistence of a habitat created by large scale rice farming at the centre of The Gambia that is inimical to *Anopheles gambiae* s.s. coupled with ecological differences East and West of this habitat was investigated. Also investigated was the origin of insecticide resistance in The Gambia. Population genetic structure of *An. gambiae* s.s. in The Gambia and Senegal was investigated using 15 microsatellite loci; ten on chromosome 3 and 5 on X chromosome. Results revealed strong distance-limited dispersal that was broken down by ecological barrier that separated individuals into coastal and inland populations in a similar fashion as identified in an earlier study. Populations were significantly differentiated as estimated by F_{ST}, 0.05 (95% CI 0.03 – 0.08). Variation in local selection acting at the *Vgsc*-1014F locus in populations East and West of The Gambia is responsible for the stark difference in insecticide resistance profiles between the two populations. Significant population differentiation between Senegalese and Gambian populations, $F_{ST} = 0.08$ (95% CI 0.05 – 0.11, $p < 0.001$), indicated that the high insecticide resistance in the East of The Gambia are at least not recent immigrants from Senegal and that they could have arrived earlier but been subjected to intense selection pressures or to a lesser extent represent *de novo* mutations. The results further highlight the roles of ecological adaptation in limiting gene flow in a species that is thought to be segregating in this region. Understanding the origin of insecticide resistance and the factors affecting its spread are important for malaria control in this country that is intensifying malaria control activities to move toward pre-elimination era.

5.1 Introduction

The tropical weather and climatic conditions in sub-Saharan Africa (SSA) create favourable ecological conditions for the growth and development of pathogens and their vectors (Deshmukh, 1986) causing immense public health and agricultural challenges. Despite these generally suitable climatic conditions, different malaria vectors have developed preferences for varying ecological niches (Service, 1982). Even in locations where different malaria vectors exist in sympatry, small-scale spatial and/or temporal differences can lead to adaptation to micro-niches favouring growth, development and survival of different vectors.

In West Africa, where it is not uncommon to find different malaria vectors living in sympatry, adaptation to various ecological niches has been observed. For example, *An. coluzzii* and in some cases *An. arabiensis* commonly occupy permanent to semi-permanent breeding habitats such as rice paddies and long standing flood waters (Diabaté et al., 2008). Whilst, *An. gambiae* s.s. apparently prefer temporary rain-fed pools of water that quickly dry whenever rainy seasons are over. Differences in favoured ecological conditions have also been observed among members of the *An. funestus* group where salinity affects larval growth between species (Koekemoer et al., 2014).

This niche specialization can lead to reproductive isolation where individuals from particular environments reproduce assortatively (Schluter, 2000). Over time, driven by diversifying selection, the gene flow between populations from divergent environments may reduce and ultimately cease (Rundle and Nosil, 2005) resulting in pronounced genetic structure or ecological speciation (Shafer and Wolf, 2013).

Ecological speciation may be expedited in situations where physical separation by a geographical feature like a lake, river, mountain or vegetation or a man-made physical barrier reduces gene flow further (Shafer and Wolf, 2013, Grant, 1999, Funk et al., 2005, Su et al.,

2003). The Great Rift Valley in east Africa has been demonstrated to play an important role in limiting gene flow between *An. gambiae* s.s. in Kenya resulting in population sub-structuring (Kamau et al., 1999, Lehmann et al., 1999).

Geographic distance, in the absence of apparent major topographic barriers, between populations can also bring about genetic differences in malaria vectors (Donnelly et al., 1999, Lehmann et al., 2003). Conversely ecological adaptation is thought to be a major driver of genetic differentiation in *An. gambiae* s.s. and *An. arabiensis* in Kilombero valley in Tanzania (Ng'habi et al., 2011) and in *An. gambiae* s.s. in Ghana and Burkina Faso (Yawson et al., 2007). Individual populations, herein defined as

'*groups of individuals of the same species living in close proximity that any member of the group can potentially mate with any other member*' (Waples and Gaggiotti, 2006)

may be evident from genetic differentiation, i.e. population genetic structure. Where populations, regardless of environment, are confronted by an alien stress like insecticides, as is the case of malaria vectors, all individuals would benefit from a trait that confers survival or fitness advantage against the stressor.

In the absence of strong reproductive barriers beneficial mutations may spread by migration and selection, but since having such traits is very key for survival, significant reproductive isolation, where present, may be temporarily relaxed (Norris et al., 2015), resulting in introgression of adaptive genetic material (Clarkson et al., 2014, Weill et al., 2000). Alternatively, beneficial traits may occur independently via *de novo* mutations in different populations and in different geographic areas (Pinto et al., 2007, Santolamazza et al., 2015).

In The Gambia, *An. gambiae* s.s. populations in the east have been found to exhibit genetic differentiation from those in the west of the country (Caputo et al., 2014), possibly due to: (1)

differential ecological adaptation to eastern and western habitats and/or; (2) an extensive rice farming region in the centre of the country that is inimical to *An. gambiae* s.s. creating a discontinuity in their distribution.

However, this rice farming does not extend South/North into Senegal and therefore possibilities of population mixing might still occur from the periphery. The ecological conditions brought about by rice farming in this Central River Region (CRR) provides suitable breeding habitats for *An. coluzzii* but not for *An. gambiae s.s.* (Caputo, 2008)(chapters 2 &4; Opondo *et al* 2016) creating discontinuity between those populations as suggested by Caputo et al (Caputo et al., 2014).

Historically, in The Gambia, two major insecticide classes have been used in public health; pyrethroids on LLINs and DDT for IRS until 2015 when the GNMCP switched to bendiocarb. Data from chapters two (Opondo et al., 2016) and three indicate resistance is largely confined to *An. gambiae* s.s. populations from eastern Gambia where presence of *Vgsc-1014F* was shown to be a strong predictor of phenotypic resistance compared to western *An. gambiae* s.s. populations, where this mutation is at much lower frequencies (less than 8% in 2013 or 4% in 2014). It is not known whether mutants carrying the *Vgsc-1014F* in western Gambia are migrants from the east or are a result of *de novo* mutations.

The primary aim of the work was to investigate temporal persistence of genetic structure where earlier work demonstrated that populations east and west were genetically differentiated and to determine the origin of insecticide resistance in The Gambia. Three hypotheses that were not mutually exclusive were investigated: that the insecticide resistance observed in populations from east of Gambia could: (1) arise from migrants from Senegal following a wave of selection sweeping from inland West Africa through into The Gambia; (2) be driven by local selection pressure; and/or (3) differentiation between populations could

be maintained by large scale rice farming in the middle of the country that acts as a barrier. To test these hypotheses a population genetic study of *An. gambiae* s.s. from The Gambia and eastern Senegal was carried out using microsatellite markers.

5.2 Methods

5.2.1 Study area and mosquito collection

Anopheles gambiae s.s. samples were collected during the rainy seasons of 2013 and 2014 in The Gambia while Senegalese samples were collected in 2010. In both countries, collection points were relatively close to the sampling locations, or were located within the ecological regions, identified and described by Caputo *et al* (Caputo, 2008) (Caputo et al., 2014)(**Figure 19**). Only *An. gambiae s.s.* samples were analysed in this study because of the stark contrast in *Vgsc-1014F* frequency and susceptibility to insecticides between east and western populations of The Gambia, which was absent from the primarily susceptible *An, coluzzii* and *An. arabiensis* populations (Chapter two; (Opondo et al., 2016)).

Gambian *An. gambiae* s.s. were randomly selected from samples collected from nine villages and included both resistant and susceptible individuals. The majority of the specimens had been sampled as larvae from breeding habitats and reared to adults with some arising from blood fed females that produced F1, which were reared to adults and tested for phenotypic resistance against DDT and deltamethrin. Senegalese *An. gambiae s.s.* samples were selected from specimens collected using Pyrethrum Spray Catch (PSC) indoor in 2010 from three villages Badi, Barkeyel and Wassadou and supplied by Ibrahima Dia (**Figure 19**).

For this study, the Gambian samples were randomly selected from those used in chapters two and three and since DNA had already been extracted, DNA extraction was thus done only on Senegalese samples. The extraction method, species identification and screening for molecular markers of resistance were similar to the protocol used in chapters two and three.

5.2.1.1 Ecological zones of study villages

The nine villages in The Gambia and the three villages in Senegal were located across different ecological habitats. The variations in the type of soil, water, agriculture and vegetation cover were previously analysed using 'landscape approach' in ArcMap (Version 9.2;ESRI) software and lead to the following definitions of Eco geographic zones (Caputo, 2008):

(1) Lower River Area (LRA) that lies in the west of the country and extends up to about 200 kilometres inland. Mangrove landscape is common with relatively mild temperatures and higher rainfall suitable for rain dependent agriculture. Agricultural production of groundnuts, millet and rice and cattle keeping is practiced. Brackish and fresh water environments proximal to the more arid Sudan Savannah areas are common especially towards the coast on the northern side of the river Gambia. In addition, open woodland Savannah/farmland covers most of the free-draining sand laterite soil in this northern side of the river.

(2) Central River Area (CRA) that is characterized by flat Sudan Savannah landscape where presence of fresh water floodplains allow year round rice cultivation (rice-field landscape). (3) The more arid Upper River Area (URA) with free draining alluvial soils and characterized by tree, shrub Savannah and cultivated areas. Wood coupled with cultivation landscape is common and (4) Eastern Area (EA) characterized by shrub/woodland savannah and large cultivated areas almost replacing natural forest ecosystem (**Figure 19**) (Caputo, 2008).

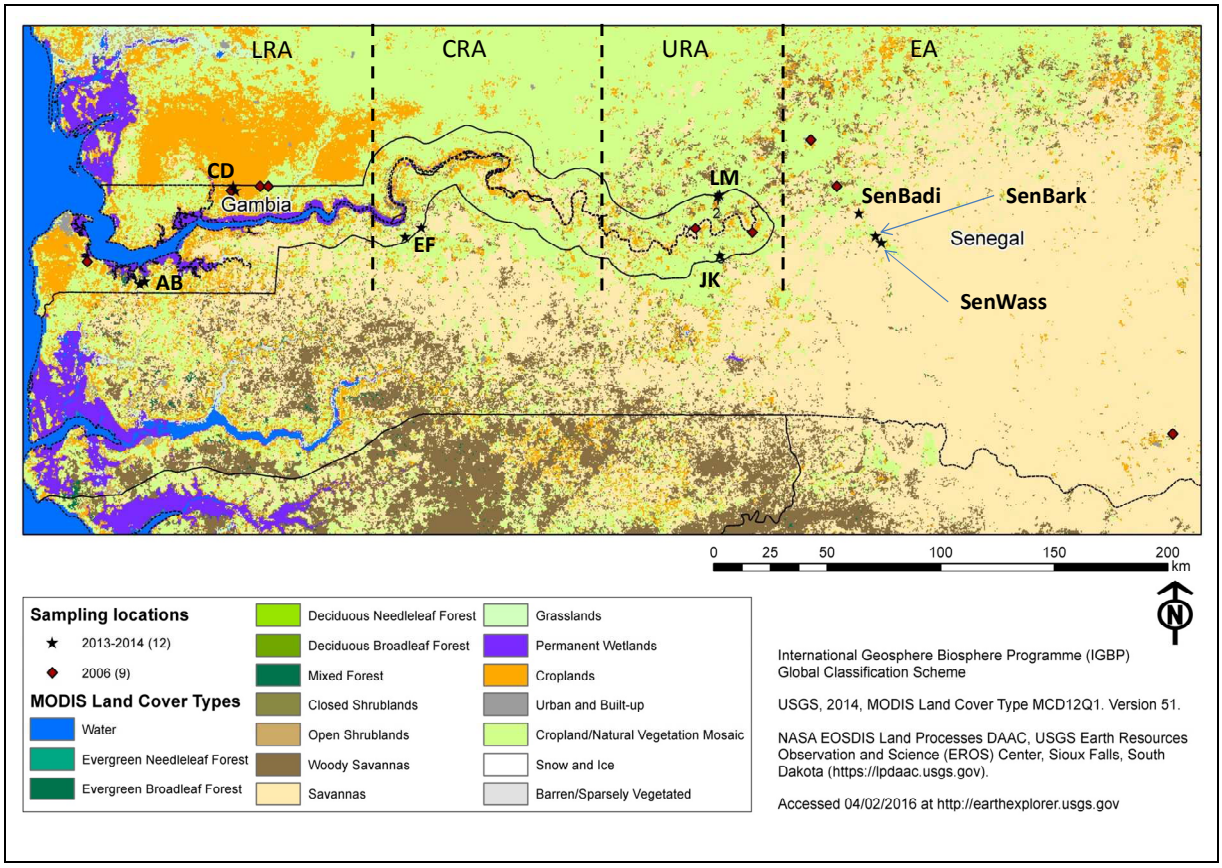

Figure 19: Ecological map representing major ecosystems, ecological zones separated by dashed lines and land cover types in Senegal and The Gambia showing sampling locations of *An. gambiae* s.s. used in this study. Black stars represent twelve sampling locations for samples collected in transmission seasons of 2013 and 2014 for Gambian samples and 2010 for Senegalese samples. Red diamond points represent sampling locations in 2006 by Caputo et al (Caputo et al., 2014). LRA represent Lower River Area, CRA – Central River Area, URA – Upper River Area and EA – Eastern Area. Letters AB represent sampling site 1, CD site 2, EF site 3, JK site 4, LM site 5, SenBadi site 6, SenBark site 7 and SenWass site 8.

5.2.2 Microsatellite genotyping

Fifteen microsatellite loci, 10 on chromosome 3 and five on chromosome X, were used to investigate population structure (**Table 12**). All the loci were used by an earlier study within the same geographical region (Caputo et al., 2014). The difference between the earlier and

the present study is that only 5 loci on chromosome X were analysed in the present study rather than 10 as the former. Chromosome three was chosen because it is less impacted by chromosomal inversions which have been implicated in adaptation to environments (Ayala et al., 2014). Two loci, one on chromosome X (XND6U4) and the other in chromosome three (X45C1) failed to amplify properly in the multiplexed PCR (see below) in all the samples and were hence excluded from all the analyses.

Table 12: Microsatellite systems used in population structure analysis of *An. gambiae* s.s. samples from The Gambia and Senegal.

Locus	Chromosome (location)	Repeat motif & no.	Allele size range	Dye	Primer sequence, (F,R)	TA (°C)
AGXH678	X (5D)	(TC)7	133-173	Vic	CCTCTCCCCAGAATCGGTAC AAGAGCAGAAACAACCGCA G	61.8
XND6U2	X(6)	(CA)13	250-300	Vic	TTGTTGCTCGGCTTGAAGTA GAAGGAATCGAGGGTGCTCT	60.5
XND5D1	X(5D)	(CA)11	120-180	Vic	GGAAACCGACACCACAAAG TGCCATTGAATGATGATGATG	60
XND5C1	X(5C)	(GT)10	230-280	6-fam	TCGCTTCGACAAATCATCAC GGGCGAAAATTCGTACAGAG	60.5
AG3H242	3L (45B)	(GT)8	49-109	Ned	TTCATTTCCACCGCAGCTGC GGCGACACTCAATCCTTCC	61.5
AG3H128	3R (29C)	(GT)21	87-137	Pet	CGGGACGGCTAGATAAAGCG CCGGGCGACATAACCCACCC	63
AG3H93	3R (29A)	(GT)4+7	130-210	6-fam	TCCCCAGCTCACCCTTCAAG GGTTGCATGTTTGGATAGCG	63
AG3H119	3R (31(B)	(GT)6	165-250	Ned	GGTTGATGCTGAAGAGTGGG ATGCCAGCGGATACGATTCG	61.8
AG3H555	3R (32C)	(GT)8	70-120	6-fam	GCAGAGACACTTTCCGAAAC TGTCAACCCACATTTTGCGC	58
AG3H249	3R (30D)	(GT)15	90-160	Ned	ATGTTCCGCACTTCCGACAC GCGAGCTACAACAATGGAGC	61.8
AG3H59	3R (29D)	(GT)9	93-153	Vic	CCCCTATTAAACCCTGGACG TGTTGTTGCCCTGCGTTACC	61.1
AG3H577	3L (42A)	(GT)16	70-150	Pet	TTCAGCTTCAGGTTGGTCTC GGGTTTTTTGGCTGCGACTG	59
AG3H758	3L (43A)	(GT)11	65-145	6-fam	TGATTTGCCAGTTCTGCCAG GTGATTGGAGTGGCTAGTGG	59.2

5.2.2.1 Designing PCR multiplexes

The 15 loci were amplified in three multiplexes of five loci each, designed using Multiplex manager (version 1.2) (Holleley and Geerts, 2009). Multiplex manager takes into account the

expected size range of different loci, their annealing temperatures, and the colour of dye used (or expected to be used) to group loci into single reactions. Based on the annealing temperature range provided, three annealing temperatures were calculated; 59°, 60.1° and 62.2° C. Each locus was then allocated into any of the 3 groups based on how close its annealing temperature was to the new calculated ones. Grouping was made in such a way that loci with smaller differences in size were excluded from the group or assigned a contrasting dye colour to avoid ambiguity in reading scores (**Figure 20**,**Figure 21**).

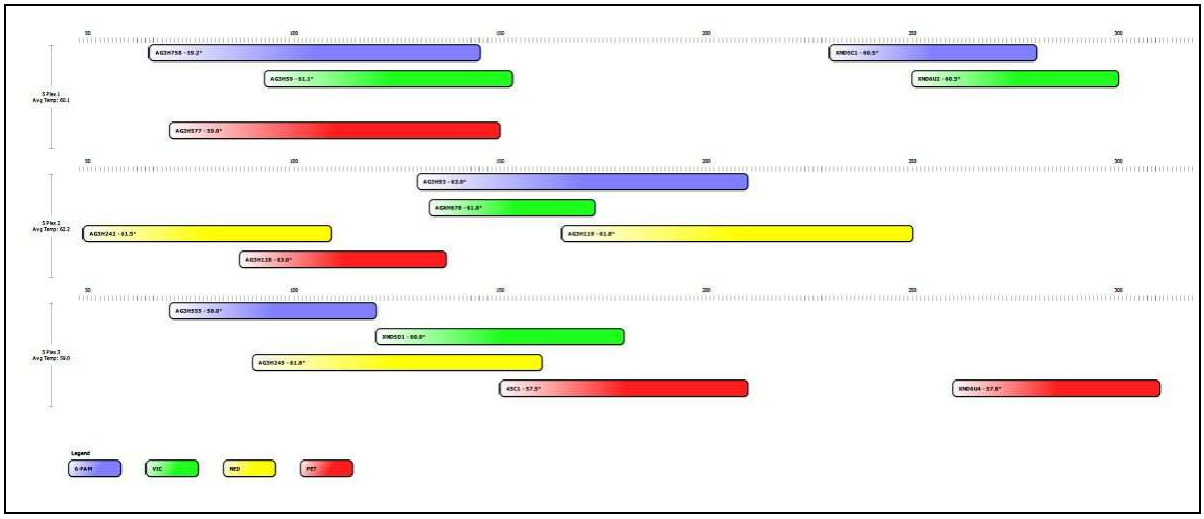

Figure 20: Pictorial representation of results from multiplex manager showing three groups of multiplexes of five loci each. Group one is represented by plex 1 (top row) with annealing temperature of 60.1°, followed by plex 2 (middle row) with 62.2° and plex 3 (last row) with 59.0° Celsius. Colours represent the dye colour in the Forward Primer used to identify individual loci within the multiplex.

5.2.2.1.1 Primer preparation for multiplexes and reaction conditions

For each of the multiplexes 10 µl each of 100 µM reverse and forward (fluorescent labelled with a dye) primers were mixed together and totalled with water to make a primer mix of 500 µl by volume. The QIAGEN® Multiplex PCR kit (Qiagen) was used to amplify multiplexes. A total PCR reaction volume of 18.25 µl was made by mixing 6.25 µl of MM solution, 1.25 µl of primer mix, 8.75 µl of RNase free water and 2 µl of DNA sample into a single reaction tube (**Table 13**). These were amplified according to standard PCR thermal profile with variations only at the annealing temperature for each of the multiplexes (**Table 14**)

Table 13: PCR reaction components and volume

Reagents	Single reaction tube (µl)
MM solution	6.25
Master primer mix	1.25
Water	8.75
DNA	2
Total	18.25

Table 14: PCR thermal profile for multiplexes

Temperature °C	Time (hh:mm:ss	Comment
95	00:05:00	
95	00:00:30	
60.1	00:00:30	Annealing temperature, adjusted per multiplex
72	00:00:30	
72	00:00:30	
10	00:10:00	

5.2.2.2 Genotyping alleles

Fragment analysis of amplified PCR products was performed by capillary electrophoresis by Macrogen, South Korea. Electropherogram data of each amplified multiplex were imported into GeneMapper® 5 (Thermo Fisher Scientific) and peaks of amplified products representing allele sizes (**Figure 21**) were manually scored to give raw allele sizes in decimal points.

Raw sizes were then binned to give allele sizes. Although earlier authors reported that alleles at all the loci varied by two repeat units (Caputo et al., 2014), single base pair differences were observed in the present study. The binned allele sizes were then carefully checked for genotyping errors due to null alleles, large allele dropouts and stutter peaks using MICRO-CHECKER software (Van Oosterhout et al., 2004). Overall, MICRO-CHECKER suggested presence of null alleles due to homozygous excess but this was distributed across all populations so analyses of population differences initially included all the 13 amplified loci. Scoring error due to stuttering was suggested in four loci, but this was an erroneous signal caused by presence of alleles with one repeat unit; AG3H93, AG3H119, AG3H758 and

AG3H555 (**Appendix 23**). All electropherograms were checked manually to confirm that true allele peaks were scored.

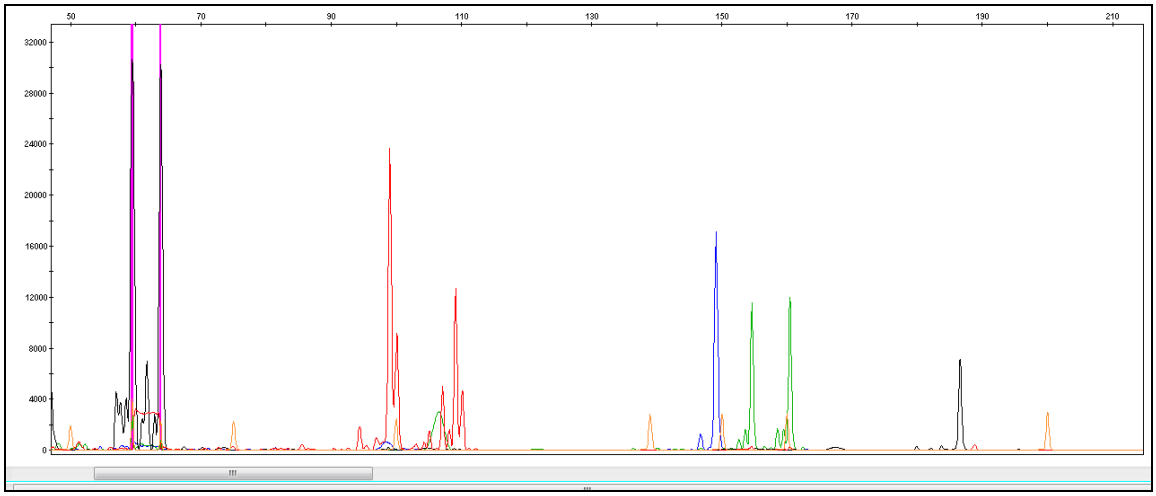

Figure 21: A section of graphical view of allele size peaks of one sample as displayed in GeneMapper® software. Colours represent different amplified loci (four in this case)

5.2.3 Statistical analysis

For the Gambian samples, a sample site consisted of individuals from paired villages in each region due to their very close proximity, while for Senegal; a village sampling point represented a sample site because villages were much further apart. Thus, Gambian sample sites were; AB, CD, EF, JK, LM, and Senegalese, SenBadi (Badi), SenBark (Barkeyel) and SenWass (Wassadou).

The populations were analysed for microsatellite diversity per locus per population by computing allele richness Rs, which corrects for variation in sample size by prediction of the number of alleles present if all samples were the size of the smallest one (El Mousadik and Petit, 1996), expected heterozygosity He, (Nei, 1987) and inbreeding coefficient, F_{IS} using FSTAT (version 2.9.3.2) (Goudet, 2001).

Significance of $F_{IS,}$ i.e. departure from Hardy Weinberg Equilibrium (HWE), was assessed by randomization tests in FSTAT. Linkage Disequilibrium (LD) between each pair of loci in each population was assessed using Fisher's Exact tests in GENEPOP ON THE WEB (Rousset, 2008). For LD, since high genetic differentiation in admixed populations could lead to higher LD, individuals were re-assigned to the different clusters identified following TESS cluster analysis (see below) and LD analysis repeated.

Unless otherwise stated, where multiple pairwise tests were performed, the significance level was adjusted by sequential Bonferroni procedure (Holm, 1979) or strict Bonferroni correction in FSTAT. Since null alleles may affect population differentiation, FreeNA (Chapuis and Estoup, 2007) was used to generate synthetic genotypes after correcting for null alleles and F_{ST} derived from it compared with F_{ST} derived from original dataset.

Bayesian cluster analysis was initially performed using STRUCTURE v.2.3.4 (Pritchard et al., 2000) with 100,000 burn in runs followed by 500,000 iterations repeated 10 times for each value of K from 1 to 10. The optimum number of clusters was determined using the Evanno method (Evanno et al., 2005) implemented in STRUCTURE Harvester (Earl, 2012).

The optimum number of clusters (K) inferred is that K at which ΔK has the largest value. The solution was not consistent, with K_{OPT} interchangeably being either 2 or 3. Following this, the spatially explicit Bayesian algorithm implemented in TESS, that takes into account

geographical location by incorporation of a prior which assumes that closer neighbours are more likely to be similar than those further away. The analysis was run using a no admixture model with 50,000 burn in sweeps and 100,000 MCMC iterations repeated 10 times for each value of K_{MAX} = 2-9 and the optimal number of clusters evaluated based on Deviance Information Criterion (DIC) values.

The effect of isolation by distance (IBD) on the overall genetic differentiation and differentiation due to *Vgsc-1014F* was assessed using simple and partial Mantel tests (PMT). For the resistance locus, wild allele was assigned size value of 100 while mutant (Phenylalanine - *F*) assigned 200 such that heterozygote was assigned size of 100 and 200.

Two FST matrices were derived from the putative neutral microsatellites and also from the *Vgsc-1014F* marker and their correlation with pairwise geographic distance assessed in zt software (Bonnet and Van de Peer, 2002) and in ncf package (version 1.1-6) in R statistical software (Version 3.1.2). For PMT, to test the effect of *Vgsc-1014F* on genetic differentiation and whether geographical structure limited dispersal of the *Vgsc-1014F* allele, the correlation between geographical distances and F_{ST} derived from neutral microsatellites while controlling for F_{ST} from *Vgsc-1014F* was tested.

Also investigated was whether the resistance allele was restricted by population structure by investigating if the correlation between the *Vgsc-1014F* allele and geographic distance improved while controlling for genetic differences between populations. Since the location of populations on either side of the river and clustering might affect IBD analysis, these were subsequently controlled for. Thus, following STRUCTURE analysis, the 3 clusters identified were used to generate binary dummy variable matrices such that a value of one (1) was given between pairs of populations within a cluster and zero (0) when they were from different

clusters. Similarly, comparisons of populations on the same side of the river were given a value of 1 and of 0 when they located different sides of the river.

Genetic distances as estimated by Da (Nei 1987) and differentiation by Theta (Weir and Cockerham, 1984) were computed and neighbour joining dendogram built for both. The tree that best explained the variation in the data i.e having the highest value of R2 was chosen. Hence, genetic distance using Da gave the best fit and thus was used to build an un-rooted neighbour joining dendogram in TreeFit (version 1.2) (Kalinowski, 2009) and visualized in TreeView software (version 1.6.6) (Page, 1996).

To identify loci potentially behaving non-neutrally, LOSITAN (Antao et al., 2008) was applied under the infinite allele mutation model with 200,000 simulations and 'Neutral' F_{ST} delimited at the 99% confidence interval. Three loci, AG3H577, XND6U2 and XND5C1 gave a higher F_{ST} for their level of heterozygosity than would be expected under neutral evolution, and therefore neighbour joining dendograms with and without the three loci were compared. Reliability of the final tree was assessed by running 1000 bootstrap pseudo-replicates over loci within TreeFit.

To test the hypothesis of local selection acting on the *Vgsc-1014F* (*kdr*) locus, F_{ST} values derived from overall microsatellites and that from the *kdr* locus were compared. Since high heterozygosity at microsatellites suppresses F_{ST} estimates (Hedrick, 2005), microsatellite data were re-coded using RecodeData software (version 0.1) (Meirmans, 2006) and new standardized F_{ST}' value computed for this new dataset in FSTAT. This F_{ST}' is an estimate of the maximum F_{ST} that could have been obtained given the observed level of homozygosity. Simply, the standardized F_{ST} was obtained by calculating F_{ST} from the original data-file and then divided by F_{ST} from the recoded data-file.

Standardized F_{ST} estimate from overall microsatellites was then compared with F_{ST} from *Vgsc-1014F*. In addition, this standardized differentiation measure (G'_{ST}), was measured directly using an online algorithm, software for the measurement of genetic diversity (SMOGD) (Crawford, 2010) and compared to that from *Vgsc*-1014F. Further, the *kdr* locus was coded into numbers and combined with the neutral microsatellites and the whole dataset run in LOSITAN selection detection software.

Determination of possible migrants carrying the *kdr* allele was assessed using GENECLASS (Piry et al., 2004), GENEALEX (Peakall and Smouse, 2006) and results from both were then compared to TESS output at K_{MAX}=6. First, in GENECLASS, using Paetkau et al protocol (Paetkau et al., 2004), an individual was assigned to be either a resident or migrant using likelihood method where p-value was likelihood in test population divided by maximum likelihood of any population (likelihood_home/max_likelihood). An individual was assigned a population if the p-value was less than 0.05. Membership into any of the populations, expressed as probability of assignment into a population, was also generated using the same algorithm. To check for consistency, population assignment using the same algorithm (Paetkau et al., 1995, Paetkau et al., 2004) but implemented in GENALEX (Peakall and Smouse, 2006, PE, 2012) was also performed.

5.3 Results

A total of 451 *An. gambiae* s.s. from 8 sites were genotyped at 13 microsatellite loci. Polymorphism across the 13 loci as indicated by allele richness (Rs) varied between 1.71 at locus AG3H242 to 8.68 at locus AG3H128, with expected heterozygosity (He) varying between 0.64 (AG3H242) and 0.92 (AG3H128). Thirty-seven out of 104 tests indicated deviation from HWE, all due to heterozygote deficits. Loci AG3H758 and AG3H93 contributed 30% of these tests followed by loci AG3H555 (11%). Thirty percent of significant tests came from population LM, followed by CD (27%) and AB (22%) (**Table 15**).

For LD, 214 tests out of 642 were in LD ($P<0.05$) and after Bonferroni correction, 113 remained significant. There was no correlation between number of locus pairs significantly in LD and the number of tests that were not in HWE across populations suggesting that this reduced heterozygosity may not be as a result of Wahlund effect.

Table 15: Genetic diversity of microsatellite loci estimated by F_{IS} per locus by population.

	Sampling site							
loci	AB	CD	EF	JK	LM	BADI	Barkeyel	Wassadou
AG3H242	**0.364**	**0.216**	0.134	0.191	**0.164**	0.333[a]	NA	0.199
AG3H128	0.058	**0.192**	0.029	0.061	**0.140**	0.261	0.343[a]	0.036[a]
AG3H93	**0.571**	**0.630**	**0.395**	0.317	**0.481**	0.337	0.077[a]	**0.433**
AGXH678	0.119	**0.181**	**0.328**	0.055	**0.364**	-0.015	0.348[a]	0.143
AG3H119	**0.426**	**0.296**	0.183	0.053	**0.266**	0.316	0.200[a]	0.251
AG3H555	**0.469**	**0.314**	-0.055[a]	-0.029[a]	**0.168**	-0.158[a]	0.158[a]	**0.431**
AG3H249	-0.001	0.038	0.014[a]	-0.132	0.037[a]	-0.234[a]	0.111[a]	0.169
XND5D1	0.014	0.039	0.313	-0.086[a]	0.073	-0.147[a]	0.193[a]	0.058
AG3H59	0.133	**0.153**	0.149	0.258	**0.205**	-0.091[a]	0.304	0.158
AG3H577	**0.201**	**0.342**	0.178	-0.057[a]	**0.445**	0.115[a]	0.059[a]	0.107[a]
AG3H758	**0.574**	**0.412**	**0.491**	**0.385**	**0.527**	0.361[a]	0.298[a]	**0.578**
XND5C1	**0.160**	**0.195**	0.026[a]	0.106[a]	**0.187**	0.418	0.510[a]	0.111
XND6U2	**0.557**	0.132	0.143[a]	0.294	**0.171**	**0.534**	0.360[a]	0.149

F_{IS} estimates of genetic differentiation per locus by population. [a] indicate loci that were in Hardy Weinberg Equilibrium (HWE) before Bonferroni correction. Those in bold indicate loci that were NOT in HWE after Bonferroni correction.

Relative to neutral expectations, two loci on the X chromosome (XND6U2, XND5C1) and one locus on chromosome 3 (AG3H577) showed an excess of differentiation for their level of heterozygosity suggesting they could be under selection (**Figure 22**). The genetic differentiation across the whole data set, as measured by global F_{ST} was 0.053 (95% CI: 0.03-0.076) and when the three outlier loci were excluded, there was a non-significant reduction in the estimate of genetic differentiation, $F_{ST, 95\% CI}$=0.036 (0.024-0.049). Subsequent analyses proceeded with all loci but some analyses were repeated with the three loci removed (see

below). Different loci gave varying levels of differentiation as measured by F_{ST} with XND6U2 and AG3H577 being the most differentiated (**Figure 23**).

There was highly significant (<0.001) pairwise population differentiation (F_{ST}) in all but two comparisons that involved Senegalese populations where Barkeyel was compared with either Badi or Wassadou (**Table 16**). The correlation between pairwise F_{ST} estimated from synthetic data corrected for null alleles in FreeNa and the original dataset was very high indicating little impact of null alleles in the observed differentiation (Mantel test, r = 0.99, p = 0.001) (**Appendix 24**).

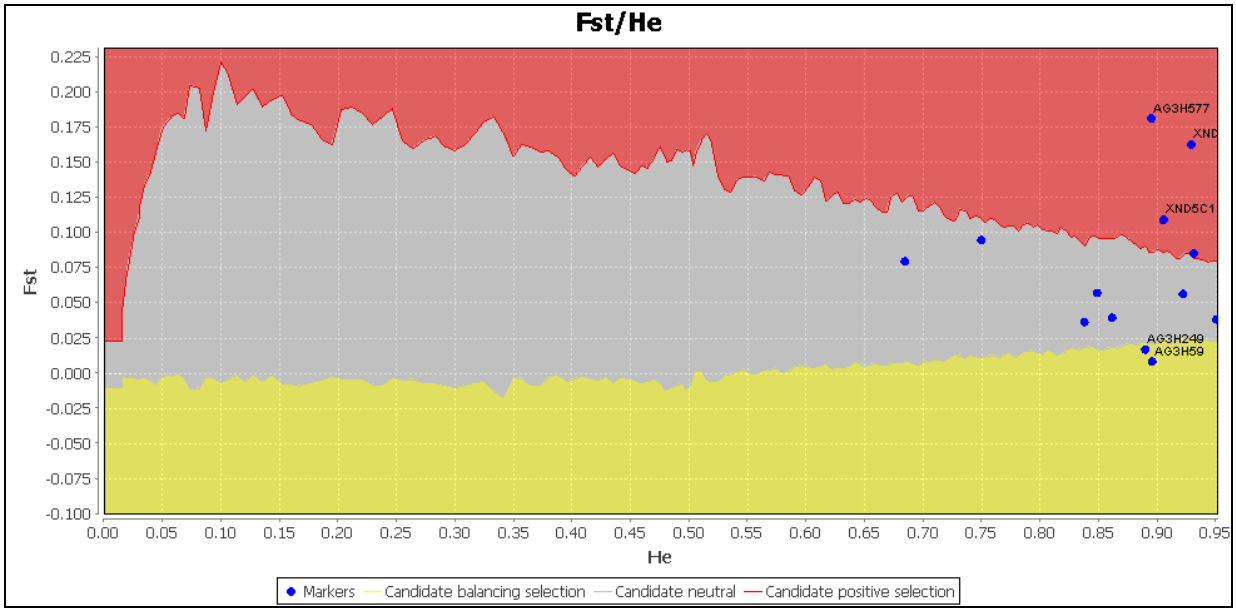

Figure 22: Neutrality tests of microsatellite loci among populations using LOSITAN. Genetic differentiation (F_{ST}) between loci plotted against expected Heterozygosity (He). The grey area represents the 99% confidence envelope expected under neutrality. The red zone represents loci under selection while yellow region represent loci potentially under balancing selection. The truncated loci within the red zone is XND6U2.

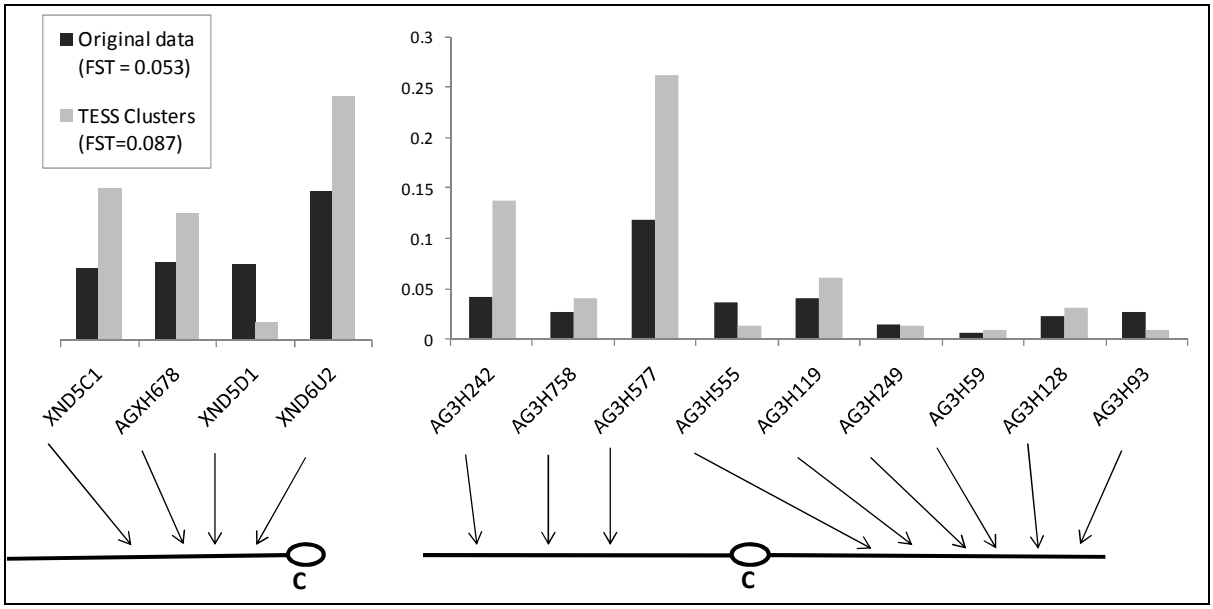

Figure 23: Genetic differentiation, F_{ST}, per locus across populations derived from original sample sites (dark bars) and TESS clusters (at $K_{MAX} = 6$) (gray bars). Three X chromosome loci (to the left) and two in chromosome 3 (to the right) show greater differentiation especially when estimated for the TESS clusters (grey bars). The grey bars indicate that clusters identified by TESS are more differentiated than original data although not significant. Arrows point to physical mapping of the loci within the X chromosome and chromosome 3. C denotes centromere positions on each chromosome.

Table 16: Pairwise population differentiation, F_{ST}, estimated from 13 loci.

FST	AB	CD	EF	JK	LM	Badi	Barkeyel
CD	0.039						
EF	0.0819	0.0588					
JK	0.093	0.0745	0.0428				
LM	0.0498	0.0308	0.0364	0.0304			
Badi	0.0919	0.0808	0.0926	0.0841	0.051		
Barkeyel	0.1163	0.1042	0.1257	0.1155	0.0776	**0.044**[a]	
Wassadou	0.0737	0.0679	0.0713	0.0664	0.0349	0.0168	**0.062**[a]

Bold F_{ST} values with [a] denote non-significant pairwise test.

5.3.1 *Anopheles gambiae* s.s. sub-structure

A non-spatial model, implemented in STRUCTURE, without a priori geographical location was unable to identify a single optimal number of clusters where two or three clusters were identified interchangeably. In the first case of two clusters, there was western population composed of individuals from coastal ecosystem (AB, CD) and inland ecosystem consisting of individuals from (EF, JK, LM, SenBadi, SenBark, SenWass). Where three clusters were identified, the coastal populations were further split into two; south coast (AB) and north coast populations (CD) with the remaining populations being clustered into one group of inland populations (**Figure 24**, **Appendix 25**).

Since biological interpretation of the number of clusters, K, may not be straightforward (Pritchard et al., 2000), the optimal number of clusters chosen was 2 (K=2) and was a more parsimonious explanation to the population clusters observed in STRUCTURE where each cluster represented a mix of individuals from different sites and this corresponded to the PCoA (**see Figure 29**) where coastal populations (AB and CD) were separated from putatively inland populations (**Figure 24**).

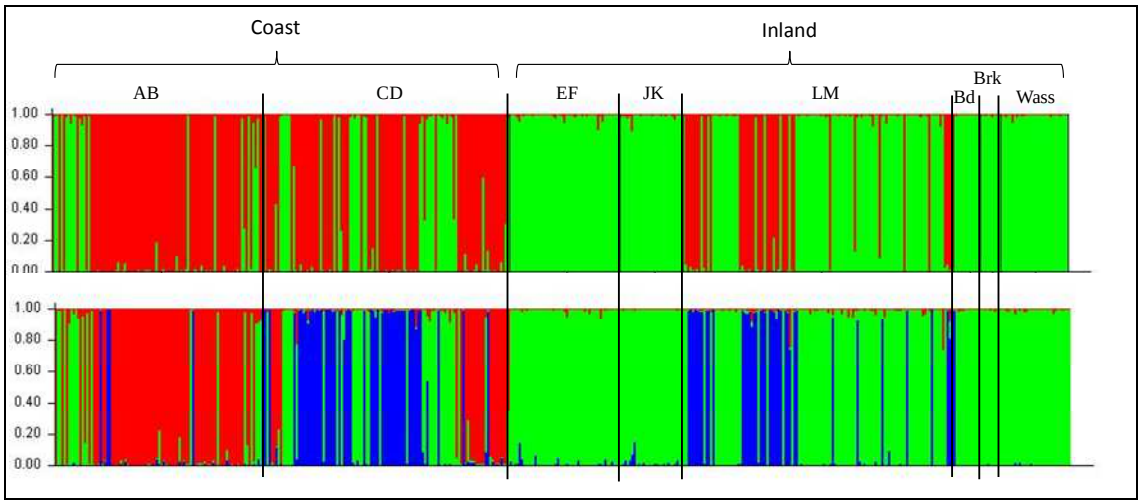

Figure 24: Population structure of *An. gambiae* s.s. sampled in 2010, 2013 and 2014 based on 13 loci showing coastal and inland populations. Bars represent proportion of assignment of individuals to clusters as inferred by STRUCTURE at K_{OPT} = 2 (top) and 3 (bottom).

A spatially explicit model implemented in TESS revealed six clusters, K_{MAX} =6, although there was no clear point of inflection to determine K_{MAX} (**Figure 25**) and therefore the optimum number of clusters was inferred visually both from DIC and cluster membership. From the average cluster membership in each K_{MAX} from 2 to 9, the major divisions identified in K_{MAX} =2 remained unchanged but were delineated as distinct clusters with

increases in K up to K=6 beyond which the major divisions begun to show changes in pattern of divisions indicating an unstable solution (**Figure 26**). The optimum K arrived at was thus, $K_{MAX} = 6$.

Out of the six clusters, five represented major clusters corresponding to sampling locations however; one cluster was composed of populations from JK and EF. The last sixth cluster, labelled as cluster3, were individuals that split from south coastal population, AB and inland populations (**Figure 26**). TESS cluster membership indicates presence of both pure and mixed populations within the study area (**Figure 26**).

Individuals were reassigned to the new clusters identified in TESS to form new populations where LD was assessed. A total of ninety tests were found to be in LD out of 624 tests performed after Bonferroni correction. Cluster 1 representing populations from AB and CD contributed the highest number of significant tests 34 (38%) followed by those in cluster 3 representing populations from JK and EF at 29 (32%).

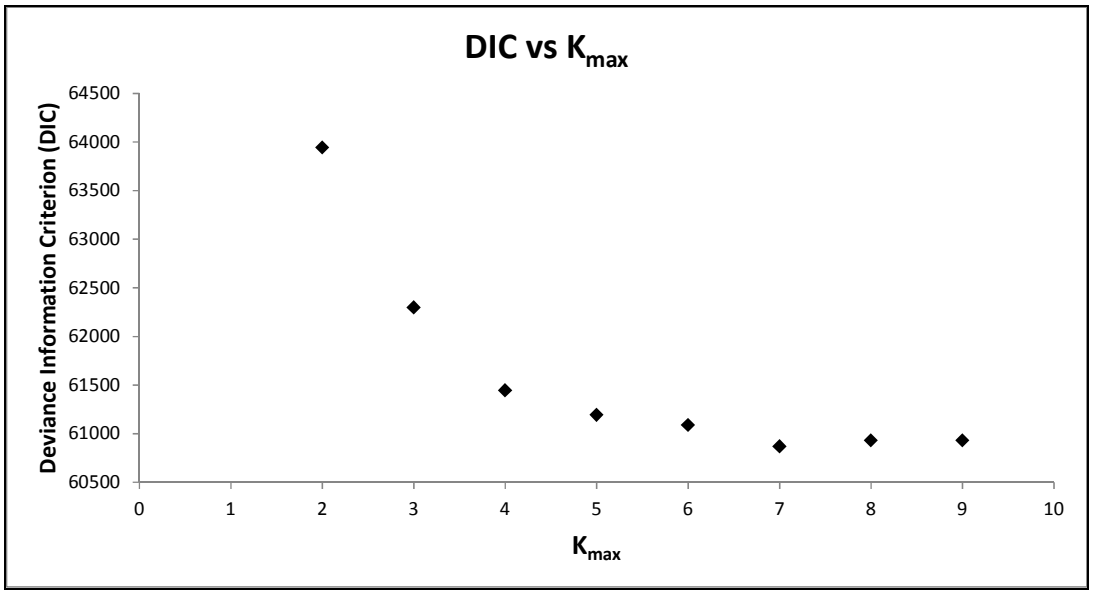

Figure 25: DIC values plotted for each K_{max} following TESS run with no admixture model.

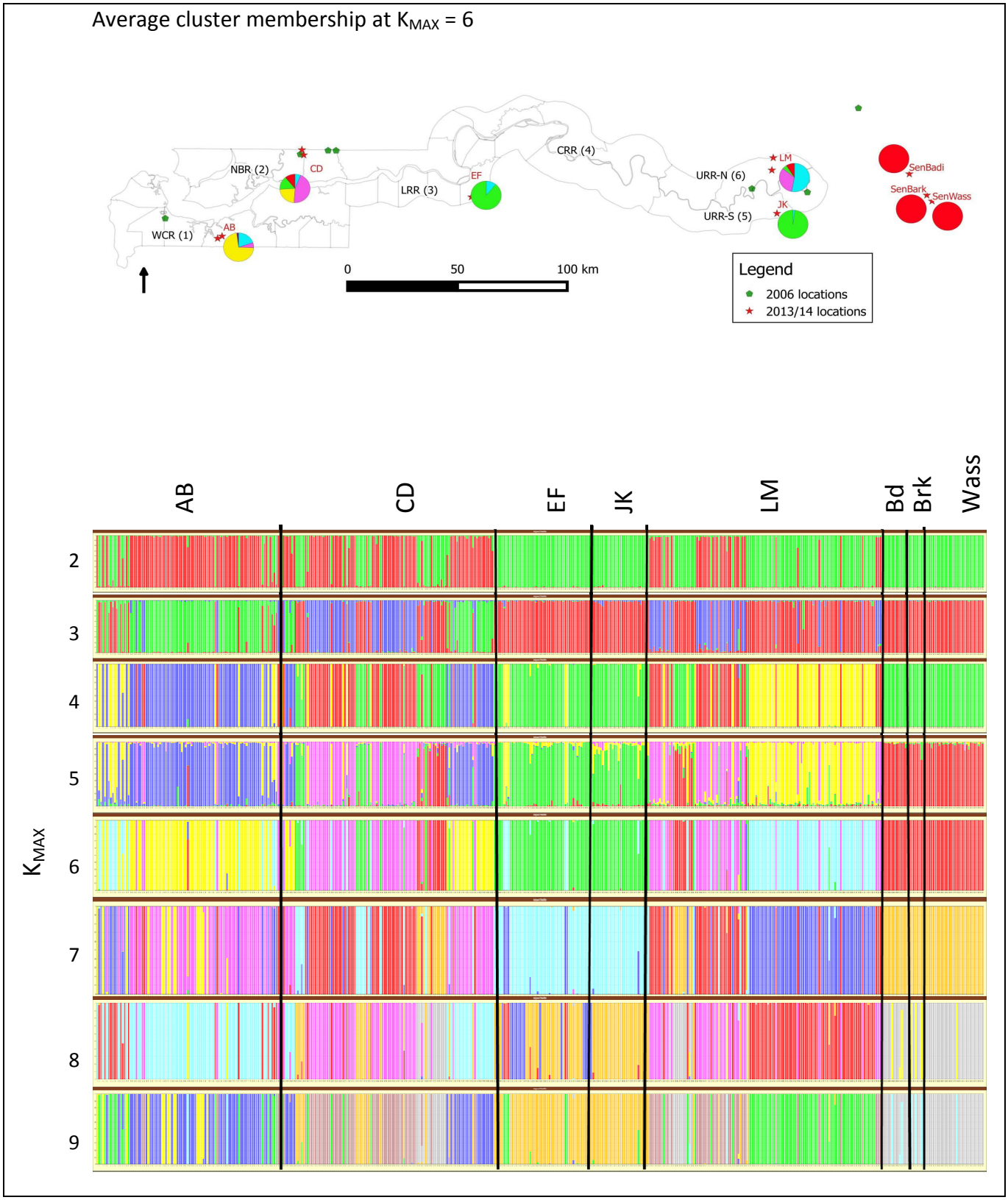

Figure 26: Population structure of *An. gambiae s.s.* sampled in 2010, 2013 and 2014. Pie charts represent average cluster membership of individuals into the six clusters identified in TESS at K_{MAX} = 6. Bd refers to populations from Badi, Brk-Barkeyel and Wass-Wassadou.

5.3.2 Hypothesis testing

Strong differentiation between eastern Gambia and Senegalese samples (**Table 16**) was not consistent with the first hypothesis that the high frequencies of *Vgsc-1014F* resistance allele in the east were migrants from Senegal (overall *Vgsc-1014F* frequency- 61.7% (95% CI 51.1 – 71.4)), following a wave of selection sweeping across West Africa.

Although eastern Gambia populations and those from Senegal occupy an inland ecosystem, distance and other factors might have been responsible for limiting gene flow between them. Simple and partial Mantel tests showed that genetic differentiation based on microsatellites was limited by distance even when controlling for which side of the river populations were sampled from (**Table 17**).

Mantel tests suggested that *Vgsc-1014F* allele frequency distributions were marginally (non-significantly) restricted by geographical distance, but with no obvious effect of 'neutral' genetic population structure since there was a reduction in correlation in PMT controlling for pairwise microsatellite F_{ST} (**Table 17**). This suggests a moderate effect of distance limited dispersal in *kdr* distribution but the involvement of other factors in the distribution of insecticide resistance. Moreover *kdr* did not have any effect on the correlation between genetic structure and geographical distance suggesting it is not a determinant of genetic structure or gene flow (**Table 17**).

Table 17: Mantel test output showing correlations between pairwise genetic differences, $F_{ST,}$ and geographic distances for both simple and partial mantel tests.

Mantel test outputs			**Corr (r)**	**P**
Simple test (10000 permutations)	pairs			
	F_{ST} vs Geographic distance		0.5	0.0006
	Kdr vs Geographic distance		0.3	0.053
Partial mantel test		Controlling for		
	F_{ST} vs Geographic distance	Side of river	0.5	0.013
	F_{ST} vs Geographic distance	*Kdr*	0.5	0.008
	Kdr vs Geographic distance	F_{ST}	0.24	0.09
	Kdr vs Geographic distance	Side of river	0.3	0.08

Kdr refer to *Vgsc-1014F* mutation. Corr refer to correlation coefficient

5.3.2.1 Isolation by distance

Correlation analysis of pairwise population F_{ST} and geographic distance in Mantel test showed a strong and highly significant isolation by distance (**Figure 27**). When the three loci were excluded, the correlation improved marginally, r = 0.56, p = 0.001. The correlation between pairwise FST values with and without the three loci was high, Mantel test, r = 0.9, p = 0.001.

To determine whether differentiation was influenced by the side of the river Gambia from which they were collected as potentially suggested visually (**Figure 28**), a partial Mantel test was conducted controlling for side of the river as a dummy variable. The side in which populations were located relative to the river did not have an impact (no improvement of correlation) in the IBD observed above (**Table 17**).

Whether the population structure was limited by *kdr* was also investigated. Results indicated that population structure was not limited by *kdr* (**Table 17**). In addition, following cluster analysis in STRUCTURE using K=3, the correlation between pairwise population differences (pairwise F_{ST}) and geographic distances improved marginally (results not shown). Despite the geographic distance influencing the genetic variation, clear separation of populations was not evident apart from coastal and inland populations (**Figure 29**).

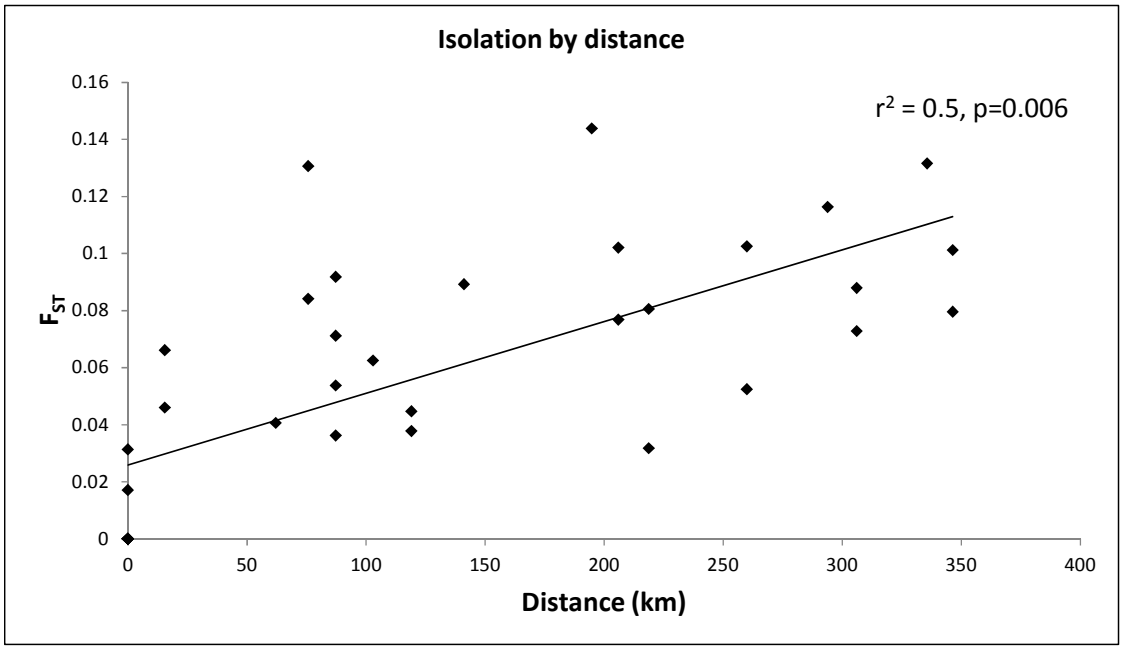

Figure 27: Isolation by distance (IBD) plot in *An. gambiae* s.s. P-value is from Mantel test.

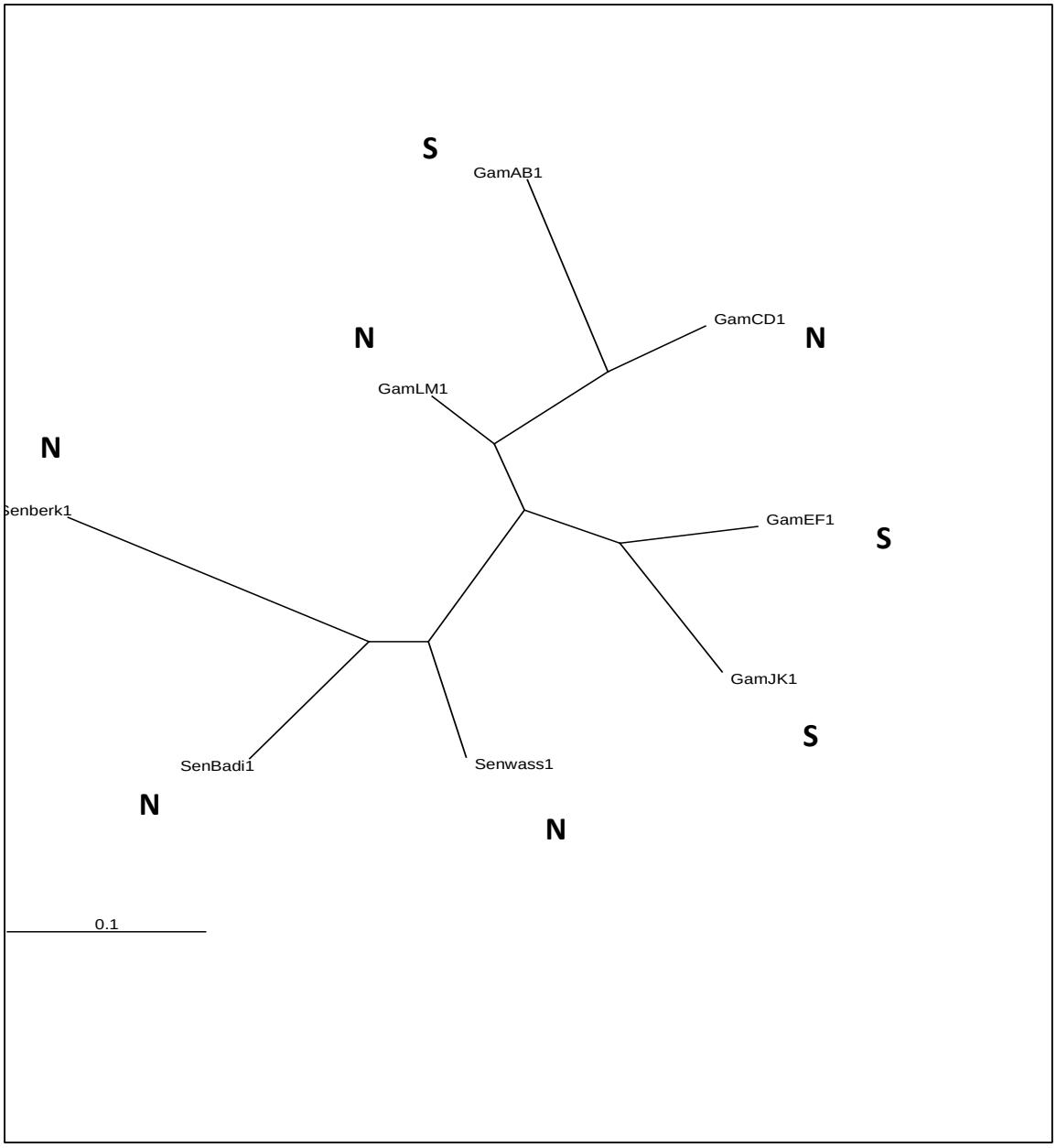

Figure 28: Unrooted neighbour joining dendogram of sample populations produced using the genetic distance, Da (Nei 1987) in TreeFit and visualized in TreeView. Populations with alphabetically more similar labels are closer. Letter N- denotes sampling locations on the Northern side of the river Gambia while S refers to those on the South.

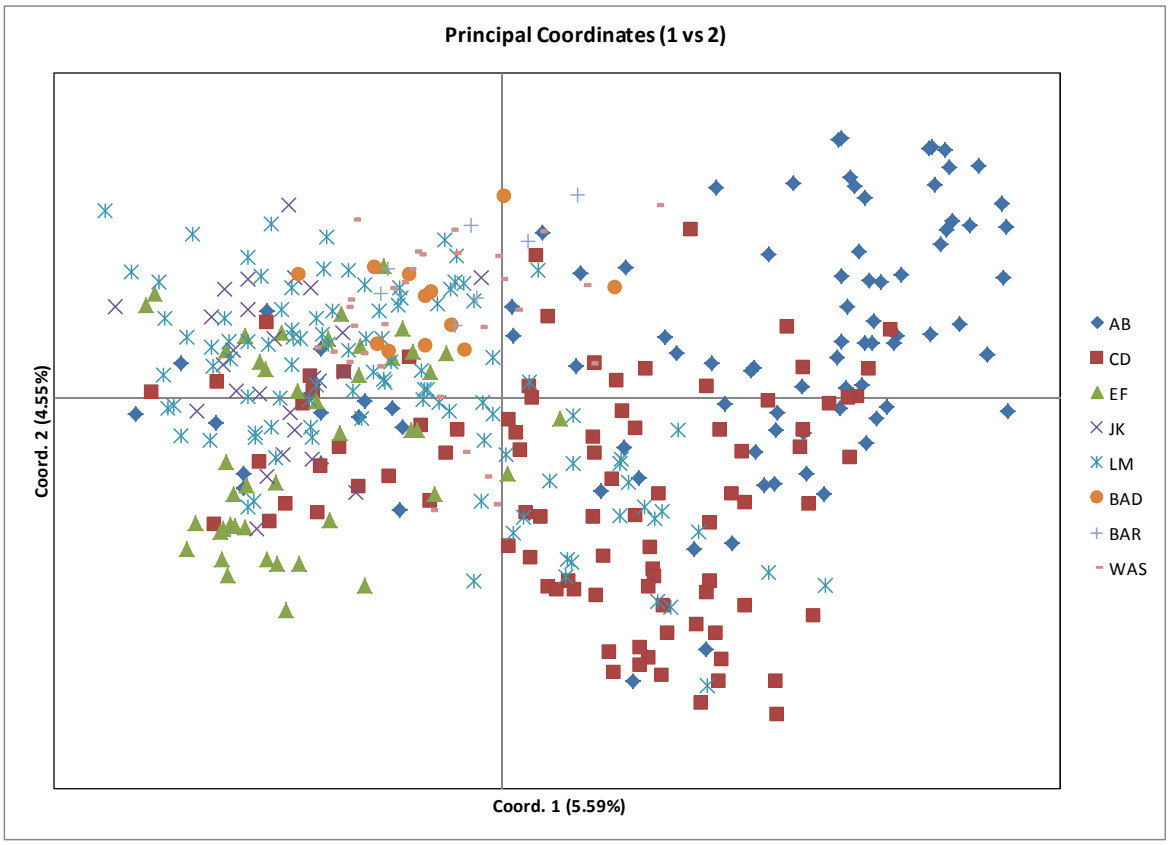

Figure 29: Principal coordinate analysis (PCoA) plot from the original dataset with populations corresponding to sampling locations. Population AB and CD represent coastal populations while the rest lie within the inland ecosystem. Three axes explain 13.72% of variation within the data.

The second hypothesis that resistance could be driven by local selection pressure was also tested. The G'_{ST} derived from *Vgsc-1014F* was compared with heterozygosity adjusted G_{ST} (G'_{ST}) (Hedrick, 2005). Global genetic differentiation of *L1014F* allele was higher, G'_{ST} 0.635 (95% CI 0.592 – 0.707) than that from neutral microsatellites, G'_{ST}, 0.357 (95% CI 0.236 – 0.477). G'_{ST} was not significantly different when the three outlier loci identified in the original data were included (G'_{ST} 0.357 (95% CI 0.236 – 0.477) or not (G'_{ST} 0.242 (95% CI 0.168 – 0.345), and remained significantly lower than *kdr* G'_{ST}.

Support for selection acting on the *kdr* locus was also provided by LOSITAN analysis of *kdr* which indicated the extreme level of differentiation compared to microsatellites (**Figure 30**). Finally suggestion of local selection is further supported by the lack of genetic structure limiting the distribution of this resistance allele (**Table 17**). Taken together these results strongly implicate local selection as a force driving *L1014F* variation within The Gambia.

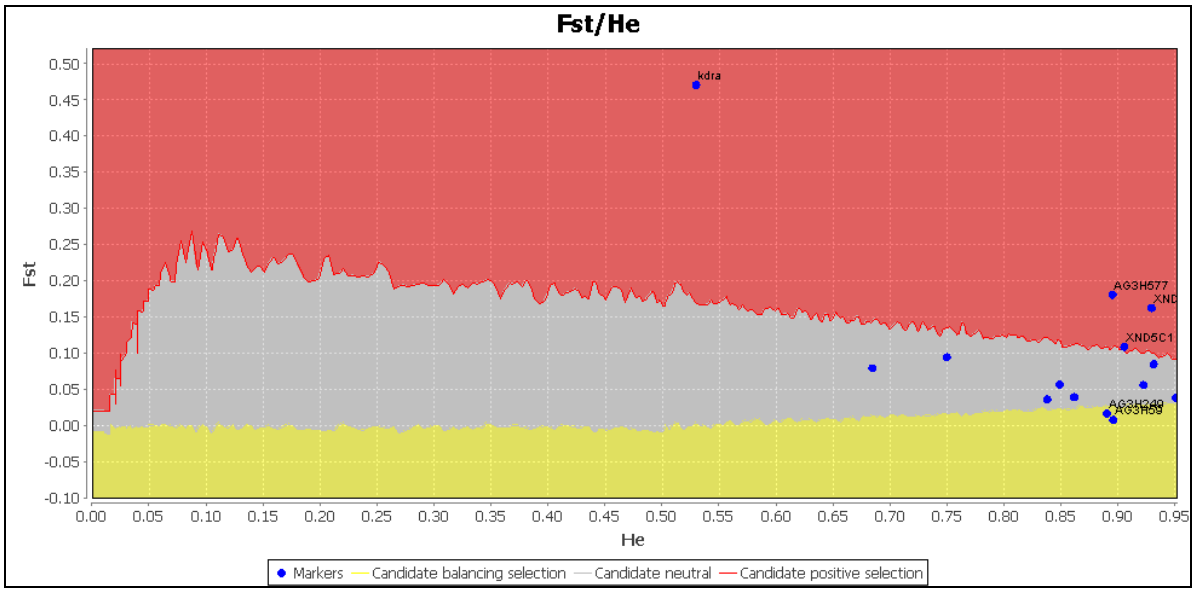

Figure 30: Neutrality tests of 13 microsatellite loci together with *kdr* (*kdr*a in figure) among populations using LOSITAN. Genetic differentiation (F_{ST}) between loci plotted against expected Heterozygosity (He). The grey area represents the 99% confidence envelope expected under neutrality. The red zone represents loci under selection while yellow region represent loci potentially under balancing selection. the truncated loci within the red zone is XND6U2.

An earlier study hypothesised that large scale rice farming in the middle of the country could have been responsible for and maintaining genetic differentiation between east (inland) and western (coastal) populations (Caputo et al., 2014). In the present study, despite the strong IBD influencing the genetic differences within *An. gambiae* s.s., temporal persistence in the

spatial pattern of genetic structure where populations are grouped into two major clusters representing coastal (western) and inland populations suggest existence of a stable barrier to gene flow which probably represents the *An. coluzzii* dominated CRR. This barrier could also be maintained by differences in ecology on both sides of the CRR.

5.3.3 Migrant identification

To examine whether the relatively rare carriers of *kdr* in western populations were more likely to be immigrants, possible migrants were identified in GENECLASS and GenAlex. A total of 284 (63%) out of the total 451 samples were identified as residents while the remaining 167 (37%) were identified as possible migrants and the same individuals were identified in the two programs.

Although resistance to insecticides was observed much more in the second year in coastal populations (chapter 3), only four carriers of *Vgsc-1014F* allele in coastal populations were identified as migrants with two of these coming from URR and EA (**Table 18**). The other 30 mutants that carried the resistance allele in the coastal populations were considered as residents (**Table 18**, **Appendix 26**).

Table 18: Insecticide resistance traits of individuals identified as possible first generation migrants. Column heads represent populations from which migrants originated and shows whether they carried *kdr* or not (wild). Row names indicate the population where the migrant moved into and thus sampled from.

			Migrant arriving from															
			AB		CD		EF		JK		LM		SenBadi (Badi)		SenBark (Barkeyel)		SenWass (Wassadou)	
Into (site)	N	Migrant no. (%)	wild	*kdr*	wild	*kdr*	wild	*kdr*	wild	*kdr*	wild	*kdr*	wild	*kdr*	wild	*kdr*	wild	*kdr*
AB	94	21 (22.3)			7		6				6				2			
CD	109	46 (42.2)	15	2			14		2		5	1	1				9	1
EF	49	18 (36.7)			1				15	12	1	1					1	1
JK	28	4 (14.3)					4	4										
LM	120	61 (50.8)	2	2	29	27	6	6	9	9			2	2	2	2	9	8
BAD	12	8 (66.7)													3	2	5	4
BAR	9	4 (44.4)											1	1			3	3
WASS	30	5 (16.7)											4	2	1			

5.4 Discussion

This study highlights distance limited dispersal as major factor limiting gene flow but IBD observed was broken down by ecological barrier that separated individuals into coastal and inland populations as observed in STRUCTURE analysis and this appears stable over time when compared to an earlier study in 2006 that also observed similar genetic partitioning (Caputo et al., 2014). The stability of genetic differentiation between east and western *An. gambiae* s.s. populations suggests the presence of a barrier to gene flow which probably represent the vast rice farming region in CRR dominated by *An. coluzzii* and maintained by ecological differences east and west of this CRR.

However, for *kdr*, there was only marginal effect of distance limited dispersal of the resistance allele and population structure did not have an impact in limiting its spread. The stark difference in insecticide resistance observed within Gambian populations either represent *de novo* mutations that arose due to selection pressure acting at local scale or they could have spread from elsewhere but frequency variation could be as a result of differences in local selection.

While mutation rate for eukaryotes is low (1×10^{-8}) (Lynch, 2010), four independent mutations were reported to have given rise to *kdr* alleles in Cameroon (Etang et al., 2009). Although the geographical scale was relatively larger than that observed between Gambian populations, the reduction in gene flow between populations in The Gambia brought about by ecology might support the hypothesis of *de novo* mutations. Nonetheless, a more likely source of resistance observed here could be migration since mosquito dispersal within this setting can go up to 1.5 – 2 kilometres (Thomas et al., 2013).

Isolation by Distance and gene flow

Geographical distance, physical barriers and ecological adaptation have previously been demonstrated as factors that lead to reduced gene flow between members of the *An. gambiae* complex. In the present study, distance played an important role in limiting gene flow between populations but this was broken by what appeared to be an ecological barrier that split individuals into east and western populations. The effect of distance was so strong such that a non-spatial model implemented in STRUCTURE (Pritchard et al., 2000) could not converge at a solution to assign individuals into clusters. The algorithm interchangeably gave the optimum number of clusters, K, to be 2 or 3.

For the case of K = 2, populations sampled in coastal area were put into one cluster while the second cluster represented all those sampled inland. When K = 3, the coastal populations were further split into those North and South of the river while the rest were clustered into one representing all inland sampling sites. This coastal/inland separation however was not complete with some individuals located in either ecosystems being assigned into 'wrong' cluster, whether K = 2 or 3.The temporal stability in the east/west partitioning supports the third hypothesis that differentiation between *An. gambiae* s.s. East and West of the Gambia could be maintained by large scale rice farming in the middle of the country that acts as a barrier to gene flow.

When analysed using a spatially explicit algorithm implemented in TESS, 6 clusters were identified representing 5 sampling locations that probably represented settings of varying micro-ecology. The sixth cluster however composed of individuals that split from four populations. While generally two main ecosystems representing wetter coastal and drier

inland ecosystems exist, micro-variation has been documented (Caputo, 2008) and probably could also have contributed genetic differentiation observed between sampling sites.

The influence of geographical distance in shaping pattern of genetic structure in *An. gambiae* s.s. varies with geographical scale with some studies showing greater differentiation at relatively smaller distances (Midega et al., 2010) (Kamau et al., 1998) but not at wider geographical scales (Lehmann et al., 2003).

This may be explained by the phenomenon of 'founder effect' where small number of individuals split from a larger populations to form different sub-populations. These sub-populations will carry different subsets of the genetic traits compared to the original larger population and can be differentiated at smaller spatial scales. When they are pooled together, the genetic differences disappear because they represent the overall genetic structure of the larger sub-population as is the case for *An. gambiae* s.s. in Africa (Lehmann et al., 2003).

The geographic distances between populations in this study were moderate but nonetheless genetic partitioning followed a stepping stone model (SMM) where individuals of the geographically closer are more likely to exchange genes than those further away (Slatkin, 1993). However, this was broken by the existence of an ecological barrier that separated individuals that were east and west of the country. Despite the geographic distance influencing the genetic variation, under the SMM of gene flow, clear separation of populations may not be evident especially where sampling locations are fairly continuous as in this study as indicated in the PcoA plot (**Figure 29**).

Thus, the substantial population differentiation between Gambian and Senegalese populations was not consistent with the first hypothesis that the *kdr* carriers in The Gambia were migrants

from Senegal following a wave of selection sweeping from inland West Africa. This study used samples drawn from field collections conducted in 2013 and 2014 and insecticide resistance in coastal populations was higher in 2014 compared to 2013. However, only four carriers of *kdr* in the coast were identified as immigrants with the rest of *kdr* carriers being considered as residents.

Whilst it might have been expected that they could possibly represent immigrants, the intense sampling employed in 2014 (using artificial larval breeding habitats) might have improved the probability to sample all possible populations otherwise un-sampled in the 2013. Nonetheless, many non-carriers of *kdr* were identified as immigrants but this high number could be due to strong IBD that would affect migrant identification.

The migrant identification algorithm (Paetkau et al., 2004) implemented in both GENECLASS and GeneAlex would imply that these are recent migrants but cannot say how recent they are. The results nonetheless suggest that *kdr* might have arrived in the West a while ago but because of weaker selection, has been slow to increase in frequency compared to those that arrived in the East.

Ecological adaptation and gene flow

Previously in the Gambia, analysis of microsatellite and chromosomal inversions in *An. gambiae* s.s. revealed genetic partitioning between east and western populations (Caputo et al., 2014). The authors attributed this to ecological adaptation based on the differences in the pattern of chromosomal inversions observed between populations east and west and maintained by an extensive rice farming area dominated by *An. coluzzii* that separates them.

It has been suggested that *An. gambiae* s.s. just recently colonized coastal ecosystems where they are adapting to the local coastal ecosystems and hybridizing with *An. coluzzii* and this is thought to contribute to the genetic differences observed between those coastal and inland ecosystems (Caputo et al., 2011, Weetman et al., 2012).

While high number of hybrids were not observed in samples from which specimens used in this study were drawn from (Chapters 2 and 3), previous work has shown that in this 'far west' distribution range of *An. gambiae* s.l, high levels of hybridization between *An. gambiae* s.s. and *An. coluzzii* is always reported (Oliveira et al., 2008, Weetman et al., 2012) and these hybrids have been shown to back cross more with *An gambiae* s.s. parent bringing along some genes from the sympatric member *An. coluzzii* (Nwakanma et al., 2013). Over time, these may also contribute to genetic partitioning of coastal populations from the inland populations and though not investigated here, could have contributed to the observed differentiation.

The roles of ecological adaptation in bringing about differences in genetic structure have also been observed in members of *An. gambiae* s.s. from Ghana and Burkina Faso where populations that habited mangrove vegetation typical of coastal ecosystem were differentiated from those found in the savannah zones, and to a lesser extent from those in the forest ecozone (Yawson et al., 2007). Chromosomal inversions though not analysed in this study are always known to be important in ecological adaptation by members of the *Anopheles* genus (Ayala et al., 2014, Dobzhansky and Dobzhansky, 1937).

Indeed, just recently in 2006, differences in chromosomal arrangements were observed in members of the *Anopheles gambiae* s.l. that were sampled from coastal (west) and inland (east) ecosystems(Caputo et al., 2014). An earlier study in the same region of The Gambia

have also found genetic differences based on chromosomal inversions between populations located in the coastal ecosystem compared to the drier inland ecosystem hinting at the roles that they could play in ecological adaptation (Bryan et al., 1982).

When individuals are thus adapted to their environments such that immigrants are not favoured, reduction in gene flow leads to differences in genetic structure as has been observed in Ghana (Yawson et al., 2007). The differences in ecological adaptation therefore provide support of the existence of a barrier that further maintains the reduced gene flow between populations East and West of The Gambia.

Analysis of heterozygosity

The high number of loci that were not in in HWE and the high number of pairwise tests between them that were in LD suggested that either there was high levels of inbreeding (selection) within populations or were due to grouping of gene pools termed as 'Wahlund effect' or presence of null alleles (Callen et al., 1993).

The 'Wahlund effect' arise when, say in a site, there exists sub populations and during sampling not all populations are sampled and thus these individuals though representing different sub-populations that are in HWE, they are treated as one population (Wahlund, 1928). In this study, lack of correlation between number of loci not in HWE and the number of loci pairs in LD per population suggested that 'Wahlund effect' may not have been responsible for the excess homozygotes observed.

The presence of null alleles is common to microsatellite loci within *An. gambiae* s.l (Donnelly et al., 1999, Lehmann et al., 2003) but may not have a major impact on observed differentiation (Yawson et al., 2007). While null alleles were observed in this study, their

presence had no influence in the genetic differentiation observed as indicated by a strong significant correlation in pairwise population differentiation derived from synthetic alleles (corrected for null alleles in FreeNa (Chapuis and Estoup, 2007)) and unadjusted original data.

Though null alleles result in excess homozygosity, they are not expected to result in LD and when both are observed as is the case in this study, excess inbreeding than would be expected by chance is inferred (Garcia de Leon et al., 1997). Inbreeding seems more plausible especially in this species that is thought to be segregating due to habitat discontinuities (Caputo et al., 2014).

Local selection and *kdr* frequency

The second hypothesis that local selection pressure could be responsible for the difference in *kdr* frequency was supported by analysis of genetic differentiation, estimated by G'_{ST}, which was significantly higher for *kdr* than for neutral microsatellites. However it is unclear what is driving the differential selection pressure.

The GNMCP until 2015 sprayed houses across the country, with exemption of certain parts of urban west coast, with DDT. In addition, mass distribution of LLINs (PermaNet® 2.0) has occurred throughout the country, the last one being in 2014 followed by routine distribution via health facilities. However, although no documented information on use of agricultural pesticides is available for this study, use of pesticides with similar modes of action may also contribute to selection pressure and have been implicated in bringing about insecticide resistance (Muller et al., 2008).

Although insecticide resistance is now wide-spread across much of SSA (Ranson and Lissenden, 2016), its origin and spread has been attributed to several factors. In West Africa, analysis of intron-1 sequence upstream of *kdr* mutation within the *Vgsc* gene revealed reduced diversity in samples drawn from multiple countries and this suggested that resistance was dispersed through migration and local selection further impacted its spread (Pinto et al., 2007). However, multiple origins arising due to variable selection pressure in varying ecological settings was reported at a smaller geographic scale in Cameroon (Etang et al., 2009).

Significant genetic differentiation between eastern *An. gambiae* s.s. and those from Senegal therefore suggest that recent and potentially ongoing migration of resistant populations into the east of Gambia is unlikely to be responsible for the high frequency of *kdr* observed but further analysis of sequence data may be needed to investigate this.

The difference in sampling time could have caused the observed genetic differentiation between Senegalese and eastern Gambia samples; but since the Senegalese samples were collected when resistance was already present in Senegal, under migration hypothesis, they were not expected to be different from Gambian samples. Besides, samples collected within the same year in the Gambia still showed significant genetic differentiation indicating that populations were actually differentiated.

Since the data suggest that local selection pressure acting at the *kdr* locus is responsible for development and spread of insecticide resistance, the GNMCP will therefore benefit immensely from the new insecticide, bendiocarb, which it started using in 2015 that will reduce the selection pressure for *kdr* and hence help in malaria control. However, because *Ace-1*-119S mutation that confers resistance to carbamates and organophosphates has been

detected in this setting, it is therefore imperative for the GNMCP to follow the guidelines outlined in the WHO Global Plan for Insecticide Resistance Management to further maintain the gains in malaria control achieved in The Gambia.

6 CHAPTER SIX: GENERAL DISCUSSION AND CONCLUSION

The three malaria vectors, *An. gambiae* s.s., *An. arabiensis* and *An. coluzzii* sampled across the Gambia varied in composition and relative proportions. Their distribution and subsequent adaptation to varying ecological niches brought about differences in their insecticide resistance traits that were further implicated in malaria heterogeneity. Resistance to DDT and deltamethrin was mainly observed in *An. gambiae* s.s. from the east of The Gambia though it varied in levels in all the villages studied.

Although differences in species proportions were observed between villages of varying transmission intensities, there was similarity in their composition as measured by similarity indices (Borg and Groenen, 2005, Yue and Clayton, 2005). As has been noted elsewhere, where different species live in sympatry, their relative contribution to observed malaria trends differ (Mbogo et al., 2003). This difference may arise from their behaviours in terms of where and when they feed, rest and even proceed to lay eggs.

The numbers of sampled blood-fed anophelines in this study were few to infer meaningful interpretation as to the preferred choice of blood meal and its effects in driving malaria heterogeneity. Nevertheless, sampled *An. gambiae* s.l. preferred to feed on humans while *An. funestus* (confined to CRR villages and sampled for the first time in The Gambia) preferred cows.

While all mosquito populations, resistant or susceptible, may prefer human blood as observed herein, the frequency of bites they inflict on susceptible human hosts may differ and thus bring about marked differences in transmission patterns (Scott et al., 2006).

Resistance of *An. gambiae* s.s. to DDT was more likely found in villages with high malaria transmission, especially those in the eastern part of the country. However, the implications of resistance in transmission heterogeneity may not be straight forward as the eastern part of The Gambia is where most resistance was observed and where malaria transmission has always been higher than in the rest of the country, suggesting that several factors may be involved (Mwesigwa et al., 2015, Thomson et al., 1994).

While noting challenges in study design (Kleinschmidt et al., 2015), further studies to establish causal relationship between insecticide resistance and malaria heterogeneity are needed. An infected resistant vector, whose chances of successfully feeding in the presence of insecticides are high (N'Guessan et al., 2007), may bring significant differences in transmission (Alout et al., 2013).

Adult mosquitoes sampled in villages that had both resistant and susceptible populations showed that both young and old resistant individuals as measured by gonotrophic stage were at equal chances of surviving or dying. This is in contrary to laboratory studies that show older resistant populations at higher risk of dying (Chouaibou et al., 2012, Rajatileka et al., 2011). This indicates that resistant vectors constantly exposed to insecticides in the wild benefit from pre-selection that allow them to live longer in the wild.

Vector longevity influences transmission most (Garrett-Jones and Grab, 1964, Garrett-Jones and Shidrawi, 1969) and if infected resistant mosquitoes survive exposure, they might increase transmission. In this study, only older (parous) vectors carried infective stages of the malaria parasite *Pf* , indicating the older resistant populations may still be fit to transmit malaria but the dataset is limited to draw any significant conclusion.

Other studies (Hemingway et al., 2013) have reported reduced parasite carriage in wild caught insecticide resistant malaria vectors but since their age distribution was not known, whether older resistant mosquitoes are also capable of transmitting malaria in the wild remains unresolved. In fact, lack of data leads to utilization of laboratory studies to estimate possible effect of age on malaria transmission (Saddler and Koella, 2015) and may not represent the true effect of the age of resistant vectors.

Although resistance has been reported across SSA (Ranson and Lissenden, 2016), there has been no direct causal relationship observed between rise in resistance and increased malaria transmission. This could be due to the overall barrier effect that bed nets (LLINS) are creating between man and mosquito, regardless of their resistance status.

In addition, the relative contribution of resistant and susceptible populations to the observed malaria burden has not been quantified, further limiting interpretation of the roles that resistance may play in malaria transmission. Even if there are resistant vectors, they could bite a specific group of people whose malaria status would not change, hence constant malaria prevalence despite rising resistance.

Dissecting the factors responsible for driving malaria heterogeneity therefore will require exhaustive studies that simultaneously investigate relative contribution of all possible factors. This study being part of a larger study investigating malaria transmission dynamics in The Gambia within the same villages, combining the data and performing a pooled statistical analysis may shed some light on whether resistance is likely to contribute to heterogeneity.

The phenotypic resistance to DDT and deltamethrin observed here was mainly driven by *kdr* mutation at codon 1014 (*Vgsc*-1014F) and at lesser scales by another target site mutation,

Vgsc-1575Y, and metabolic resistance marker, *Gste2-114T*. Although different mechanisms may be at play in other settings, phenotypic resistance may still ensue. A recent study has also implicated *kdr* in driving resistance to permethrin in *An. gambiae* s.s. (Musa Jawara *personal communication*) although other insecticide resistance mechanisms were not investigated.

In scenarios where a particular mutation has a very strong association with phenotypic resistance as observed for *Vgsc*-1014F in *An. gambiae* s.s. in this study, it may be plausible to utilize only genetic mutation to monitor (Weetman and Donnelly, 2015) and make decisions on the how best to redesign operational malaria control both for resistance and malaria (WHO, 2012). This is particularly useful where mutation is fixed in the population and sampling a sufficient number of specimens for phenotypic determination is logistically challenging, as is most likely to be for most NMCPs.

Population migration, adaptation and malaria transmission

This study reveals how populations of one species, *An. gambiae* s.s., isolated by distance and occupying different ecological niches can have both genetic and phenotypic differences driven by prevailing local environmental factors. The *Vgsc*-1014F mutation in the eastern *An. gambiae* s.s. approached fixation while for those in the west it was barely less than 10% in frequency.

The GNMCP is aiming to further supress the already relatively low malaria transmission with the aim of eliminating malaria from the Gambia. However, the emergence and spread or development of *de novo* resistance hinders the elimination goals. At present, population migration and interbreeding within The Gambia seem to be restricted by geographical

distance further maintained by ecological factors in those settings and thus the spread of resistance may not be rapid.

The selection pressure for *kdr* may have decreased after GNMCP switched from using DDT to bendiocarb for IRS since 2015. However, GNMCP should follow the GPRIM plan on insecticide resistance management to ensure that resistance against bendiocarb does not spread.

In conclusion, the composition and the relative proportions of the different malaria vectors in space and time and their subsequent adaptation to varying ecological niches bring about differences in their behaviours and susceptibility to insecticides. Consequently, the differences appear to be other crucial components that drive heterogeneity in malaria transmission especially in settings where different malaria vectors exist in sympatry like in The Gambia. In order to adapt and target control interventions to specific foci, it is fundamental to establish the type of vectors present, their behaviours and susceptibility to insecticides.

Limitations of the study and suggestion for further work

This study highlights vector related drivers of malaria heterogeneity. However, some villages that were classified as having high malaria in the previous year had changed in the following year (chapter 3). This highlights how the variable nature of malaria transmission between years can make understanding the factors driving heterogeneity difficult to study. Further studies conducted in more than 8 pairs of villages and for longer periods of time will be more useful.

Also, to study explicitly how resistant populations contribute to transmission heterogeneity, it is important that vectorial capacity components of resistant vectors are established, crucially, their longevity in the wild and susceptibility to *Plasmodium* parasites in addition to when and where they bite. The Gambia may be suitable for such studies because of the strong effect that *kdr* has on survival so any *An. gambiae* s.s. carrying the mutation is assumed as resistant. However, just like in this study, collecting enough samples to draw conclusions might be a challenge so intense sampling will be required together with an additional study in a different setting.

As a preliminary study, in chapter 4, limited data suggest older resistant vectors are important in malaria transmission but age was only limited to 1-parous. Future studies which seek to measure longevity should utilize new technology like infrared (Sikulu et al., 2010) to measure chronological age and also determine gonotrophic age beyond 1-parous.

In relation to the existence of malaria hotspots, in the future it will be important to establish whether resistant populations are more likely to bite humans with specific characteristics and thus might be an important group that control programmes may target especially in low transmission settings.

Future malaria transmission models should incorporate insecticide resistance parameters estimated from empirical studies to better inform malaria control programmes. Furthermore, modelling the spread of resistance between populations and how it affects malaria control will help in designing optimal insecticide resistance management strategies.

REFERENCES

Ahmad, M., Denholm, I. & Bromilow, R. H. 2006. Delayed cuticular penetration and enhanced metabolism of deltamethrin in pyrethroid-resistant strains of *Helicoverpa armigera* from China and Pakistan. *Pest Management Science,* 62**,** 805-810.

Allison, A. C. 1954. Protection afforded by sickle-cell trait against subtertian malarial infection. *British Medical Journal,* 1**,** 290.

Alonso, P. L., Brown, G., Arevalo-Herrera, M., Binka, F., Chitnis, C., Collins, F., Doumbo, O. K., Greenwood, B., Hall, B. F. & Levine, M. M. 2011. A research agenda to underpin malaria eradication. *Plos Medicine,* 8**,** e1000406.

Alout, H., Ndam, N. T., Sandeu, M. M., Djégbe, I., Chandre, F., Dabiré, R. K., Djogbénou, L. S., Corbel, V. & Cohuet, A. 2013. Insecticide resistance alleles affect vector competence of *Anopheles gambiae* ss for *Plasmodium falciparum* field isolates. *PLoS One,* 8**,** e63849.

Antao, T., Lopes, A., Lopes, R. J., Beja-Pereira, A. & Luikart, G. 2008. LOSITAN: a workbench to detect molecular adaptation based on a FST-outlier method. *BMC bioinformatics,* 9**,** 1.

Antonio-Nkondjio, C., Fossog, B. T., Ndo, C., Djantio, B. M., Togouet, S. Z., Awono-Ambene, P., Costantini, C., Wondji, C. S. & Ranson, H. 2011. *Anopheles gambiae* distribution and insecticide resistance in the cities of Douala and Yaounde(Cameroon): influence of urban agriculture and pollution. *Malaria Journal,* 10**,** 154-154.

Asidi, A., N'guessan, R., Akogbeto, M., Curtis, C. & Rowland, M. 2012. Loss of household protection from use of insecticide-treated nets against pyrethroid-resistant mosquitoes, Benin. *Emerging Infectious Diseases,* 18**,** 1101.

Awolola, T., Oduola, A., Obansa, J., Chukwurar, N. & Unyimadu, J. 2007. *Anopheles gambiae s.s.* breeding in polluted water bodies in urban Lagos, southwestern Nigeria. *Journal of Vector Borne Disease,* 44**,** 241-244.

Ayala, D., Ullastres, A. & Gonzalez, J. 2014. Adaptation through chromosomal inversions in *Anopheles*. *Frontiers in Genetics,* 5**,** 129.

Badolo, A., Traore, A., Jones, C. M., Sanou, A., Flood, L., Guelbeogo, W. M., Ranson, H. & Sagnon, N. F. 2012. Three years of insecticide resistance monitoring in *Anopheles gambiae* in Burkina Faso: resistance on the rise. *Malaria Journal,* 11**,** 232.

Bass, C., Nikou, D., Blagborough, A. M., Vontas, J., Sinden, R. E., Williamson, M. S. & Field, L. M. 2008. PCR-based detection of *Plasmodium* in *Anopheles* mosquitoes: a comparison of a new high-throughput assay with existing methods. *Malaria Journal,* 7**,** 177.

Bass, C., Nikou, D., Donnelly, M. J., Williamson, M. S., Ranson, H., Ball, A., Vontas, J. & Field, L. M. 2007a. Detection of knockdown resistance (kdr) mutations in *Anopheles gambiae*: a comparison of two new high-throughput assays with existing methods. *Malaria Journal,* 6**,** 111.

Bass, C., Nikou, D., Vontas, J., Williamson, M. S. & Field, L. M. 2010. Development of high-throughput real-time PCR assays for the identification of insensitive acetylcholinesterase (ace-1R) in *Anopheles gambiae*. *Pesticide Biochemistry And Physiology,* 96**,** 80-85.

Bass, C., Williamson, M. S., Wilding, C. S., Donnelly, M. J. & Field, L. M. 2007b. Identification of the main malaria vectors in the *Anopheles gambiae* species complex using a TaqMan real-time PCR assay. *Malaria Journal,* 6**,** 155.

Bass, C. C. 1920. An Attempt to explain the greater pathogenicity of *Plasmodium falciparum* as compared with other Species. *Journal of Tropical Medicine and Hygiene,* 23**,** 237-238.

Bayoh, M. N., Mathias, D. K., Odiere, M. R., Mutuku, F. M., Kamau, L., Gimnig, J. E., Vulule, J. M., Hawley, W. A., Hamel, M. J. & Walker, E. D. 2010. *Anopheles gambiae*: historical population decline associated with regional distribution of insecticide-treated bed nets in western Nyanza Province, Kenya. *Malaria Journal,* 9**,** 62.

Beier, J. C., Killeen, G. F. & Githure, J. I. 1999. Short Report: Entomological Inoculation Rates and *Plasmodium falciparum* malaria prevalence in Africa. *American Journal of Tropical Medicine and Hygiene,* 61**,** 109-113.

Bejon, P., Williams, T. N., Liljander, A., Noor, A. M., Wambua, J., Ogada, E., Olotu, A., Osier, F. H., Hay, S. I. & Färnert, A. 2010. Stable and unstable malaria hotspots in longitudinal cohort studies in Kenya. *Plos Medicine,* 7**,** e1000304.

Bergé, J., Feyereisen, R. & Amichot, M. 1998. Cytochrome P450 monooxygenases and insecticide resistance in insects. *Philosophical Transactions of the Royal Society B: Biological Sciences,* 353**,** 1701-1705.

Betson, M., Jawara, M. & Awolola, T. S. 2009. Status of insecticide susceptibility in *Anopheles gambiae* sl from malaria surveillance sites in The Gambia. *Malaria Journal,* 8**,** 10.1186.

Bhatt, S., Weiss, D., Cameron, E., Bisanzio, D., Mappin, B., Dalrymple, U., Battle, K., Moyes, C., Henry, A. & Eckhoff, P. 2015. The effect of malaria control on *Plasmodium falciparum* in Africa between 2000 and 2015. *Nature,* 526**,** 207-211.

Billewicz, W. & Mcgregor, I. A. 1981. The demography of two West African (Gambian) villages, 1951–75. *Journal of biosocial science,* 13**,** 219-240.

Bonnet, E. & Van De Peer, Y. 2002. zt: a software tool for simple and partial Mantel tests. *Journal of Statistical software,* 7**,** 1-12.

Borg, I. & Groenen, P. J. 2005. Modern multidimensional scaling*: Theory and applications*, Springer Science & Business Media.

Bousema, T., Drakeley, C., Gesase, S., Hashim, R., Magesa, S., Mosha, F., Otieno, S., Carneiro, I., Cox, J. & Msuya, E. 2010. Identification of hot spots of malaria transmission for targeted malaria control. *Journal of Infectious Diseases,* 201**,** 1764-1774.

Bousema, T., Griffin, J. T., Sauerwein, R. W., Smith, D. L., Churcher, T. S., Takken, W., Ghani, A., Drakeley, C. & Gosling, R. 2012. Hitting hotspots: spatial targeting of malaria for control and elimination. *Plos Medicine,* 9**,** e1001165.

Bousema, T., Stresman, G., Baidjoe, A. Y., Bradley, J., Knight, P., Stone, W., Osoti, V., Makori, E., Owaga, C., Odongo, W., China, P., Shagari, S., Doumbo, O. K., Sauerwein, R. W., Kariuki, S., Drakeley, C., Stevenson, J. & Cox, J. 2016. The impact of hotspot-targeted interventions on malaria transmission in Rachuonyo south district in the western Kenyan highlands: a cluster-randomized controlled Trial. *Plos Medicine,* 13.

Breman, J. G., Alilio, L. S. & Mills, A. 2004. Conquering the intolerable burden of malaria: What's new, What's needed: A summary. *American Journal of Tropical Medicine and Hygiene,* 71**,** 1-15.

Briët, O. J., Penny, M. A., Hardy, D., Awolola, T. S., Van Bortel, W., Corbel, V., Dabiré, R. K., Etang, J., Koudou, B. G. & Tungu, P. K. 2013. Effects of pyrethroid resistance on

the cost effectiveness of a mass distribution of long-lasting insecticidal nets: a modelling study. *Malaria Journal,* 12**,** 10.1186.

Brito, L. P., Linss, J. G., Lima-Camara, T. N., Belinato, T. A., Peixoto, A. A., Lima, J. B., Valle, D. & Martins, A. J. 2013. Assessing the effects of *Aedes aegypti* kdr mutations on pyrethroid resistance and its fitness cost. *PLoS One,* 8**,** e60878.

Browne, E., Frimpong, E., Sievertsen, J., Hagen, J., Hamelmann, C., Dietz, K., Horstmann, R. & Burchard, G. 2000. Malariometric update for the rainforest and savanna of Ashanti region, Ghana. *Annals Of Tropical Medicine And Parasitology,* 94**,** 15-22.

Bryan, J., Di Deco, M., Petrarca, V. & Coluzzi, M. 1982. Inversion polymorphism and incipient speciation in *Anopheles gambiae* s. str. in the Gambia, West Africa. *Genetica,* 59**,** 167-176.

Bryan, J., Petrarca, V., Di Deco, M. & Coluzzi, M. 1986. Adult behaviour of members of the *Anopheles gambiae* complex in the Gambia with special reference to An. melas and its chromosomal variants. *Parassitologia,* 29**,** 221-249.

Burkot, T., Graves, P., Cattan, J., Wirtz, R. & Gibson, F. 1987. The efficiency of sporozoite transmission in the human malarias, *Plasmodium falciparum* and *P. vivax*. *Bulletin of the World Health Organization,* 65**,** 375.

Burkot, T. R., Graves, P. M., Paru, R., Wirtz, R. A. & Heywood, P. F. 1988. Human malaria transmission studies in the *Anopheles punctulatus* complex in Papua New Guinea: sporozoite rates, inoculation rates, and sporozoite densities. *The American Journal of Tropical Medicine and Hygiene,* 39**,** 135-144.

Busvine, J. & Pal, R. 1969. The impact of insecticide-resistance on control of vectors and vector-borne diseases. *Bulletin of the World Health Organization,* 40**,** 731.

Busvine, J. R. 1978. Current problems in the control of mosquitoes. *Nature,* 273**,** 604-607.

Callen, D. F., Thompson, A. D., Shen, Y., Phillips, H. A., Richards, R. I., Mulley, J. C. & Sutherland, G. R. 1993. Incidence and origin of" null" alleles in the (AC) n microsatellite markers. *American Journal Of Human Genetics,* 52**,** 922.

Cano, J., Berzosa, P., Roche, J., Rubio, J., Moyano, E., Guerra-Neira, A., Brochero, H., Mico, M., Edu, M. & Benito, A. 2004. Malaria vectors in the Bioko Island (Equatorial Guinea): estimation of vector dynamics and transmission intensities. *Journal Of Medical Entomology,* 41**,** 158-161.

Caputo, B., Nwakanma, D., Caputo, F., Jawara, M., Oriero, E., Hamid-Adiamoh, M., Dia, I., Konate, L., Petrarca, V., Pinto, J., Conway, D. J. & Della Torre, A. 2014. Prominent intraspecific genetic divergence within *Anopheles gambiae* sibling species triggered by habitat discontinuities across a riverine landscape. *Molecular Ecology,* 23**,** 4574-4589.

Caputo, B., Nwakanma, D., Jawara, M.,Adiamoh, M., Dia, I., Konate, L., Petrarca, V., Conway, D. J. And Della Torre, A. 2008. *Anopheles gambiae* complex along The Gambia river, with particular reference to the molecular forms of An. *gambiae* s.s. *Malaria Journal,* 7.

Caputo, B., Santolamazza, F., Vicente, J. L., Nwakanma, D. C., Jawara, M., Palsson, K., Jaenson, T., White, B. J., Mancini, E., Petrarca, V., Conway, D. J., Besanky, N. J., Pinto, J. & Della Torre, A. 2011. The “far-west” of *Anopheles gambiae* molecular forms. *PLoS One,* 6**,** e16415.

Casimiro, S., Coleman, M., Hemingway, J. & Sharp, B. 2006. Insecticide resistance in *Anopheles arabiensis* and *Anopheles gambiae* from Mozambique. *Journal Of Medical Entomology,* 43**,** 276-282.

Ceesay, S. J., Casals-Pascual, C., Erskine, J., Anya, S. E., Duah, N. O., Fulford, A. J., Sesay, S. S., Abubakar, I., Dunyo, S., Sey, O., Palmer, A., Fofana, M., Corrah, T., Bojang, K., Whittle, H., Greenwood, B. & Conway, D. J. 2008. Changes in malaria indices between 1999 and 2007 in The Gambia: a retrospective analysis. *The Lancet,* 372**,** 1545-1554.

Ceesay, S. J., Casals-Pascual, C., Nwakanma, D. C., Walther, M., Gomez-Escobar, N., Fulford, A. J., Takem, E. N., Nogaro, S., Bojang, K. A., Corrah, T., Jaye, M., Taal, M., Sonko, A. A. & Conway, D. J. 2010. Continued decline of malaria in The Gambia with implications for elimination. *PLoS One,* 5**,** e12242.

Chandre, F., Manguin, S., Brengues, C., Dossou, Y. J., Darriet, F., Diabate, A., Carnevale, P. & Guillet, P. 1999. Current distribution of a pyrethroid resistance gene (kdr) in *Anopheles gambiae* complex from west Africa and further evidence for reproductive isolation of the Mopti form. *Parassitologia,* 41**,** 319-322.

Chapuis, M.-P. & Estoup, A. 2007. Microsatellite null alleles and estimation of population differentiation. *Molecular Biology and Evolution,* 24**,** 621-631.

Chareonviriyaphap, T., Prabaripai, A. & Bangs, M. J. 2004. Excito-repellency of deltamethrin on the malaria vectors, *Anopheles dirus, Anopheles swadiwongporni,* and *Anopheles maculatus,* in Thailand. *Journal of the American Mosquito Control Association,* 20**,** 45-54.

Chouaibou, M. S., Chabi, J., Bingham, G. V., Knox, T. B., N'dri, L., Kesse, N. B., Bonfoh, B. & Jamet, H. V. 2012. Increase in susceptibility to insecticides with aging of wild *Anopheles gambiae* mosquitoes from Côte d'Ivoire. *BMC infectious diseases,* 12**,** 214.

Chuquiyauri, R., Paredes, M., Peñataro, P., Torres, S., Marin, S., Tenorio, A., Brouwer, K. C., Abeles, S., Llanos-Cuentas, A. & Gilman, R. H. 2012. Socio-demographics and

the development of malaria elimination strategies in the low transmission setting. *Acta Tropica,* 121**,** 292-302.

Clarke, G. M., Anderson, C. A., Pettersson, F. H., Cardon, L. R., Morris, A. P. & Zondervan, K. T. 2011. Basic statistical analysis in genetic case-control studies. *Nature protocols,* 6**,** 121-133.

Clarkson, C. S., Weetman, D., Essandoh, J., Yawson, A. E., Maslen, G., Manske, M., Field, S. G., Webster, M., Antão, T., Macinnis, B., Kwiatkowski, D. & Donnelly, M. J. 2014. Adaptive introgression between *Anopheles* sibling species eliminates a major genomic island but not reproductive isolation. *Nature Communications,* 5**,** 4248.

Coetzee, M., Van Wyk, P., Booman, M., Koekemoer, L. L. & Hunt, R. H. 2006. Insecticide resistance in malaria vector mosquitoes in a gold mining town in Ghana and implications for malaria control. *Bull Soc Pathol Exot.,* 99**,** 400-403.

Cohuet, A., Harris, C., Robert, V. & Fontenille, D. 2010. Evolutionary forces on *Anopheles*: what makes a malaria vector? *Trends in Parasitology,* 26**,** 130-136.

Corbel, V., Akogbeto, M., Damien, G. B., Djenontin, A., Chandre, F., Rogier, C., Moiroux, N., Chabi, J., Banganna, B. & Padonou, G. G. 2012. Combination of malaria vector control interventions in pyrethroid resistance area in Benin: a cluster randomised controlled trial. *The Lancet Infectious Diseases,* 12**,** 617-626.

Corbel, V., N'guessan, R., Brengues, C., Chandre, F., Djogbenou, L., Martin, T., Akogbéto, M., Hougard, J. & Rowland, M. 2007. Multiple insecticide resistance mechanisms in *Anopheles gambiae* and *Culex quinquefasciatus* from Benin, West Africa. *Acta Tropica,* 101**,** 207-216.

Costantini, C., Ayala, D., Guelbeogo, W. M., Pombi, M., Some, C. Y., Bassole, I. H., Ose, K., Fotsing, J.-M., Sagnon, N. F. & Fontenille, D. 2009. Living at the edge:

biogeographic patterns of habitat segregation conform to speciation by niche expansion in *Anopheles gambiae*. *BMC ecology,* 9**,** 16.

Crawford, N. G. 2010. SMOGD: software for the measurement of genetic diversity. *Molecular ecology resources,* 10**,** 556-557.

Dabire, K., Baldet, T., Diabate, A., Dia, I., Costantini, C., Cohuet, A., Guiguemde, T. & Fontenille, D. 2007. *Anopheles funestus* (Diptera: Culicidae) in a humid savannah area of western Burkina Faso: bionomics, insecticide resistance status, and role in malaria transmission. *Journal Of Medical Entomology,* 44**,** 990-997.

Dabiré, K. R., Diabaté, A., Djogbenou, L., Ouari, A., N'guessan, R., Ouédraogo, J.-B., Hougard, J.-M., Chandre, F. & Baldet, T. 2008. Dynamics of multiple insecticide resistance in the malaria vector *Anopheles gambiae* in a rice growing area in South-Western Burkina Faso. *Malaria Journal,* 7**,** 188.

Dadzie, S. K., Brenyah, R. & Appawu, M. A. 2013. Role of species composition in malaria transmission by the *Anopheles funestus* group (Diptera: Culicidae) in Ghana. *Journal of Vector Ecology,* 38**,** 105-110.

David, J. P., Strode, C., Vontas, J., Nikou, D., Vaughan, A., Pignatelli, P. M., Louis, C., Hemingway, J. & Ranson, H. 2005. The *Anopheles gambiae* detoxification chip: a highly specific microarray to study metabolic-based insecticide resistance in malaria vectors. *Proceedings of the National Academy of Sciences,* 102**,** 4080-4084.

De Beaudrap, P., Nabasumba, C., Grandesso, F., Turyakira, E., Schramm, B., Boum, Y. & Etard, J.-F. 2011. Heterogeneous decrease in malaria prevalence in children over a six-year period in south-western Uganda. *Malaria Journal,* 10**,** 132.

De Zulueta, J. 1959. Insecticide resistance in *Anopheles sacharovi*. *Bulletin of the World Health Organization,* 20**,** 797.

Deshmukh, I. 1986. *Ecology and Tropical Biology*, Blackwell Scientific Publications.

Detinova, T. S. 1962. Age grouping methods in Diptera of medical importance with special reference to some vectors of malaria. *Monogr Ser World Health Organ,* 47**,** 13-191.

Dia, I., Diop, T., Rakotoarivony, I., Kengne, P. & Fontenille, D. 2003. Bionomics of *Anopheles gambiae* Giles, *An. arabiensis* Patton, *An. funestus* Giles and *An. nili* (Theobald)(Diptera: *Culicidae*) and transmission of *Plasmodium falciparum* in a Sudano-Guinean zone (Ngari, Senegal). *Journal of Medical Entomology,* 40**,** 279-283.

Diabate, A., Baldet, T., Chandre, F., Akogbeto, M., Guiguemde, T. R., Darriet, F., Brengues, C., Guillet, P., Hemingway, J., Small, G. J. & Hougard, J. M. 2002. The role of agricultural use of insecticides in resistance to pyrethroids in *Anopheles gambiae s.l.* in Burkina Faso. *American Journal of Tropical Medicine and Hygiene,* 67**,** 617-622.

Diabaté, A., Dabiré, R. K., Heidenberger, K., Crawford, J., Lamp, W. O., Culler, L. E. & Lehmann, T. 2008. Evidence for divergent selection between the molecular forms of *Anopheles gambiae*: role of predation. *BMC Evolutionary Biology,* 8**,** 5.

Diatta, M., Spiegel, A., Lochouarn, L. & Fontenille, D. 1998. Similar feeding preferences of *Anopheles gambiae* and *A. arabiensis* in Senegal. *Transactions of the Royal Society of Tropical Medicine And Hygiene,* 92**,** 270-272.

Diuk-Wasser, M. A., Toure, M. B., Dolo, G., Bagayoko, M., Sogoba, N., Traore, S. F., Manoukis, N. & Taylor, C. E. 2005. Vector abundance and malaria transmission in rice-growing villages in Mali. *American Journal of Tropical Medicine and Hygiene,* 72**,** 725-731.

Djègbè, I., Boussari, O., Sidick, A., Martin, T., Ranson, H., Chandre, F., Akogbéto, M. & Corbel, V. 2011. Dynamics of insecticide resistance in malaria vectors in Benin: first

evidence of the presence of L1014S kdr mutation in *Anopheles gambiae* from West Africa. *Malaria Journal,* 10**,** 261.

Djogbenou, L., Weill, M., Hougard, J. M., Raymond, M., Akogbeto, M. & Chandre, F. 2007. Characterization of insensitive acetyleholinesterase (ace-1(R)) in *Anopheles gambiae* (Diptera : Culicidae): Resistance levels and dominance. *Journal Of Medical Entomology,* 44**,** 805-810.

Djouaka, R. F., Bakare, A. A., Coulibaly, O. N., Akogbeto, M. C., Ranson, H., Hemingway, J. & Strode, C. 2008. Expression of the cytochrome P450s, CYP6P3 and CYP6M2 are significantly elevated in multiple pyrethroid resistant populations of *Anopheles gambiae* ss from Southern Benin and Nigeria. *BMC Genomics,* 9**,** 538.

Dobzhansky, T. & Dobzhansky, T. G. 1937. *Genetics and the Origin of Species,* Columbia University Press.

Dolo, G., Briët, O. J., Dao, A., Traoré, S. F., Bouaré, M., Sogoba, N., Niaré, O., Bagayogo, M., Sangaré, D. & Teuscher, T. 2004. Malaria transmission in relation to rice cultivation in the irrigated Sahel of Mali. *Acta Tropica,* 89**,** 147-159.

Dongus, S., Nyika, D., Kannady, K., Mtasiwa, D., Mshinda, H., Gosoniu, L., Drescher, A. W., Fillinger, U., Tanner, M. & Killeen, G. F. 2009. Urban agriculture and *Anopheles* habitats in Dar es Salaam, Tanzania. *Geospatial Health,* 3**,** 189-210.

Donnelly, M. J., Corbel, V., Weetman, D., Wilding, C. S., Williamson, M. S. & Black, W. C. 2009. Does kdr genotype predict insecticide-resistance phenotype in mosquitoes? *Trends in Parasitology,* 25**,** 213-219.

Donnelly, M. J., Cuamba, N., Charlwood, J. D., Collins, F. H. & Townson, H. 1999. Population structure in the malaria vector, *Anopheles arabiensis* Patton, in East Africa. *Heredity,* 83**,** 408-417.

Donnelly, M. J., Isaacs, A. T. & Weetman, D. 2015. Identification, Validation, and Application of Molecular Diagnostics for Insecticide Resistance in Malaria Vectors. *Trends in Parasitology,* 32**,** 197-206.

Donnelly, M. J., Mccall, P. J., Lengeler, C., Bates, I., D'alessandro, U., Barnish, G., Konradsen, F., Klinkenberg, E., Townson, H., Trape, J. F., Hastings, I. M. & Mutero, C. 2005. Malaria and urbanization in sub-Saharan Africa. *Malaria Journal,* 4**,** e12.

Du, W., Awolola, T., Howell, P., Koekemoer, L., Brooke, B., Benedict, M., Coetzee, M. & Zheng, L. 2005. Independent mutations in the Rdl locus confer dieldrin resistance to *Anopheles gambiae* and An. *arabiensis*. *Insect Molecular Biology,* 14**,** 179-183.

Earl, D. A. 2012. STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. *Conservation genetics resources,* 4**,** 359-361.

Edi, C. A., Koudou, B. G., Bellai, L., Adja, A. M., Chouaibou, M., Bonfoh, B., Barry, S., Johnson, P., Müller, P. & Dongus, S. 2014a. Long-term trends in *Anopheles gambiae* insecticide resistance in Côte d'Ivoire. *Parasites & Vectors,* 7**,** 1-10.

Edi, C. V., Djogbenou, L., Jenkins, A. M., Regna, K., Muskavitch, M., Poupardin, R., Jones, C. M., Essandoh, J., Ketoh, G. K. & Paine, M. 2014b. CYP6 P450 enzymes and ACE-1 duplication produce extreme and multiple insecticide resistance in the malaria mosquito *Anopheles gambiae*. *PLoS Genet,* 10**,** e1004236.

Edi, C. V., Koudou, B. G., Jones, C. M., Weetman, D. & Ranson, H. 2012. Multiple-insecticide resistance in *Anopheles gambiae* mosquitoes, Southern Cote d'Ivoire. *Emerging Infectious Diseases,* 18**,** 1508.

El Mousadik, A. & Petit, R. 1996. High level of genetic differentiation for allelic richness among populations of the argan tree [*Argania spinosa* (L.) Skeels] endemic to Morocco. *Theoretical and Applied Genetics,* 92**,** 832-839.

El Sayed, B. B., Arnot, D. E., Mukhtar, M. M., Baraka, O. Z., Dafalla, A. A., Elnaiem, D. E. A. & Nugud, A. H. D. 2000. A study of the urban malaria transmission problem in Khartoum. *Acta Tropica,* 75**,** 163-171.

Erhart, A., Thang, N. D., Van Ky, P., Tinh, T. T., Van Overmeir, C., Speybroeck, N., Obsomer, V., Hung, L. X., Thuan, L. K. & Coosemans, M. 2005. Epidemiology of forest malaria in central Vietnam: a large scale cross-sectional survey. *Malaria Journal,* 4**,** 58.

Ernst, K. C., Adoka, S. O., Kowuor, D. O., Wilson, M. L. & John, C. C. 2006. Malaria hotspot areas in a highland Kenya site are consistent in epidemic and non-epidemic years and are associated with ecological factors. *Malaria Journal,* 5**,** 78.

Essandoh, J., Yawson, A. E. & Weetman, D. 2013. Acetylcholinesterase (Ace-1) target site mutation 119S is strongly diagnostic of carbamate and organophosphate resistance in *Anopheles gambiae* ss and *Anopheles coluzzii* across southern Ghana. *Malaria Journal,* 12**,** 404.

Etang, J., Vicente, J. L., Nwane, P., Chouaibou, M., Morlais, I., Do Rosario, V. E., Simard, F., Awono-Ambene, P., Toto, J. C. & Pinto, J. 2009. Polymorphism of intron-1 in the voltage-gated sodium channel gene of *Anopheles gambiae* ss populations from Cameroon with emphasis on insecticide knockdown resistance mutations. *Molecular Ecology,* 18**,** 3076-3086.

Evanno, G., Regnaut, S. & Goudet, J. 2005. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Molecular Ecology,* 14**,** 2611-2620.

Fortin, A., Stevenson, M. & Gros, P. 2002. Susceptibility to malaria as a complex trait: big pressure from a tiny creature. *Human Molecular Genetics,* 11**,** 2469-2478.

Funk, W. C., Blouin, M. S., Corn, P. S., Maxell, B. A., Pilliod, D. S., Amish, S. & Allendorf, F. W. 2005. Population structure of Columbia spotted frogs (*Rana luteiventris*) is strongly affected by the landscape. *Molecular Ecology,* 14**,** 483-496.

Gallup, J. L. & Sachs, J. D. 2001. The economic burden of malaria. *The American Journal of Tropical Medicine and Hygiene,* 64**,** 85-96.

Garcia De Leon, F., Chikhi, L. & Bonhomme, F. 1997. Microsatellite polymorphism and population subdivision in natural populations of European sea bass *Dicentrarchus labrax* (Linnaeus, 1758). *Molecular Ecology,* 6**,** 51-62.

Gardiner, N. C., Biggar, J. R., Collins, W. & Nkrumah, F. 1984. Malaria in urban and rural area of southern Ghana; a survey of parasitaemia and of anti-malarial practice. *Journal of Tropical of Pediatrics,* 30**,** 296.

Garrett-Jones, C. & Grab, B. 1964. The assessment of insecticidal impact on the malaria mosquito's vectorial capacity, from data on the proportion of parous females. *Bulletin of the World Health Organization,* 31**,** 71.

Garrett-Jones, C. & Shidrawi, G. 1969. Malaria vectorial capacity of a population of *Anopheles gambiae*: an exercise in epidemiological entomology. *Bulletin of the World Health Organization,* 40**,** 531.

Gatton, M. L., Chitnis, N., Churcher, T., Donnelly, M. J., Ghani, A. C., Godfray, H. C. J., Gould, F., Hastings, I., Marshall, J. & Ranson, H. 2013. The Importance of mosquito behavioural adaptations to malaria control in Africa. *Evolution,* 67**,** 1218-1230.

Gaudart, J., Poudiougou, B., Dicko, A., Ranque, S., Toure, O., Sagara, I., Diallo, M., Diawara, S., Ouattara, A. & Diakite, M. 2006. Space-time clustering of childhood malaria at the household level: a dynamic cohort in a Mali village. *BMC Public Health,* 6**,** 286.

Geissbuhler, Y., Chaki, P., Emidi, B., Govella, N. J., Shirima, R., Mayagaya, V., Mtasiwa, D., Mshinda, H., Fillinger, U., Lindsay, S. W., Kannady, K., Castro, M. C., Tanner, M. & Killeen, G. F. 2007. Interdependence of domestic malaria prevention measures and mosquito-human interactions in urban Dar es Salaam, Tanzania. *Malaria Journal,* 6.

Ghebreyesus, T., Witten, K., Getachew, A., Yohannes, A., Tesfay, W., Minass, M., Bosman, A. & Teklehaimanot, A. 2000. The community-based malaria control programme in Tigray, northern Ethiopia. A review of programme set-up, activities, outcomes and impact. *Parassitologia,* 42**,** 255-290.

Ghebreyesus, T. A., Haile, M., Witten, K. H., Getachew, A., Yohannes, A. M., Yohannes, M., Teklehaimanot, H. D., Lindsay, S. W. & Byass, P. 1999. Incidence of malaria among children living near dams in northern Ethiopia: community based incidence survey. *BMJ,* 319**,** 663-666.

Gillies, M. 1974. Evidence for downwind flights by host-seeking mosquitoes. *Nature,* 252**,** 388-389.

Gillies, M. 1988. Anopheline mosquitoes: vector behaviour and bionomics. *Malaria: principles and practice of malariology,* 1**,** 453-485.

Gillies, M. & Wilkes, T. 1965. A study of the age-composition of populations of *Anopheles gambiae* Giles and A. *funestus* Giles in North-Eastern Tanzania. *Bulletin of Entomological Research,* 56**,** 237-262.

Gillies, M. T. & De Mellion, B. 1968. *The Anophelinae of Africa South of the Sahara (Ethiopian Zoogeographical Region)*, Johannesburg, South African Institute for Medical Research.

Gimonneau, G., Pombi, M., Choisy, M., Morand, S., Dabiré, R. K. & Simard, F. 2012. Larval habitat segregation between the molecular forms of the mosquito *Anopheles gambiae* in a rice field area of Burkina Faso, West Africa. *Medical and Veterinary Entomology,* 26**,** 9-17.

Githeko, A. K., Ayisi, J. M., Odada, P. K., Atieli, F. K., Ndenga, B. A., Githure, J. I. & Yan, G. 2006. Topography and malaria transmission heterogeneity in western Kenya highlands: prospects for focal vector control. *Malaria Journal,* 5**,** 107.

Gnmcp 2011. Malaria Indicator Survey. Banjul: National Malaria Control Programme.

Gordon, D., Finch, S. J., Nothnagel, M. & Ott, J. 2002. Power and sample size calculations for case-control genetic association tests when errors are present: Application to single nucleotide polymorphisms. *Human Heredity,* 54**,** 22-33.

Goudet, J. 2001. FSTAT, a program to estimate and test gene diversities and fixation indices (Version 2.9.3).

Grant, P. R. 1999. *Ecology and evolution of Darwin's finches*, Princeton University Press.

Greenwood, B. M., Bradley, A., Greenwood, A., Byass, P., Jammeh, K., Marsh, K., Tulloch, S., Oldfield, F. & Hayes, R. 1987. Mortality and morbidity from malaria among children in a rural area of The Gambia, West Africa. *Transactions of the Royal Society of Tropical Medicine And Hygiene,* 81**,** 478-486.

Greenwood, B. M. & Pickering, H. 1993. A malaria control trial using insecticide-treated bed nets and targeted chemoprophylaxis in a rural area of The Gambia, West Africa: 1. A review of the epidemiology and control of malaria in The Gambia, West Africa. *Transactions of the Royal Society of Tropical Medicine And Hygiene,* 87**,** 3-11.

Guerrant, R. L., Walker, D. H. & Weller, P. F. 2004. *Tropical Infectious Diseases,* London, Elsvier.

Hargreaves, K., Koekemoer, L., Brooke, B., Hunt, R., Mthembu, J. & Coetzee, M. 2000. *Anopheles funestus* resistant to pyrethroid insecticides in South Africa. *Medical and Veterinary Entomology,* 14**,** 181-189.

Harverson, G., Wilson, M. & Hall, P. 1968. Assessment of current malarial endemicity in Bathurst, Gambia. *West African Medical Journal,* 17**,** 63-67.

Hawley, W. A., Phillips-Howard, P. A., Ter Kuile, F. O., Terlouw, D. J., Vulule, J. M., Ombok, M., Nahlen, B. L., Gimnig, J. E., Kariuki, S. K., Kolczak, M. S. & Hightower, A. W. 2003. Community-wide effects of permethrin-treated bed nets on child mortality and malaria morbidity in western Kenya. *American Journal of Tropical Medicine and Hygiene,* 68**,** 121-127.

Hay, S. I., Guerra, C. A., Tatem, A. J., Atkinson, P. M. & Snow, R. W. 2005. Urbanization, malaria transmission and disease burden in Africa. *The Lancet,* 3**,** pp. 81-90.

Hedrick, P. W. 2005. A standardized genetic differentiation measure. *Evolution,* 59**,** 1633-1638.

Hemingway, J., Vontas, J., Poupardin, R., Raman, J., Lines, J., Schwabe, C., Matias, A. & Kleinschmidt, I. 2013. Country-level operational implementation of the Global Plan for Insecticide Resistance Management. *Proceedings of the National Academy of Sciences,* 110**,** 9397-9402.

Henry, M.-C., Assi, S.-B., Rogier, C., Dossou-Yovo, J., Chandre, F., Guillet, P. & Carnevale, P. 2005. Protective efficacy of lambda-cyhalothrin treated nets in *Anopheles gambiae* pyrethroid resistance areas of Cote d'Ivoire. *The American Journal of Tropical Medicine and Hygiene,* 73**,** 859-864.

Holleley, C. E. & Geerts, P. G. 2009. Multiplex Manager 1.0: a cross-platform computer program that plans and optimizes multiplex PCR. *Biotechniques,* 46**,** 511-517.

Holm, S. 1979. A simple sequentially rejective multiple test procedure. *Scandinavian Journal of Statistics,* 6**,** 65-70.

Huho, B., Briët, O., Seyoum, A., Sikaala, C., Bayoh, N., Gimnig, J., Okumu, F., Diallo, D., Abdulla, S. & Smith, T. 2013. Consistently high estimates for the proportion of human exposure to malaria vector populations occurring indoors in rural Africa. *International Journal Of Epidemiology,* 42**,** 235-247.

Huho, B. J., Ng'habi, K. R., Killeen, G. F., Nkwengulila, G., Knols, B. G. J. & Ferguson, H. M. 2007. Nature beats nurture: a case study of the physiological fitness of free-living and laboratory-reared male *Anopheles gambiae* sl. *Journal Of Experimental Biology,* 210**,** 2939-2947.

Hviid, L. 2005. Naturally acquired immunity to *Plasmodium falciparum* malaria in Africa. *Acta Tropica,* 95**,** pp. 270-275.

Ibrahim, S. S., Ndula, M., Riveron, J. M., Irving, H. & Wondji, C. S. 2016. The P450 CYP6Z1 confers carbamate/pyrethroid cross-resistance in a major African malaria vector beside a novel carbamate-insensitive N485I acetylcholinesterase-1 mutation. *Molecular Ecology,* 25**,** 3436-3452.

Ijumba, J. N., Mosha, F. W. & Lindsay, S. W. 2002. Malaria transmission risk variations derived from different agricultural practices in an irrigated area of northern Tanzania. *Medical and Veterinary Entomology,* 16**,** 28-38.

Irac 2010. Prevention and Management of Insecticide Resistance in Vectors of Public Health Importance. Insecticide Resistance Action Committee (ed.).

Jawara, M., Pinder, M., Drakeley, C. J., Nwakanma, D. C., Jallow, E., Bogh, C., Lindsay, S. W. & Conway, D. J. 2008. Dry season ecology of *Anopheles gambiae* complex mosquitoes in The Gambia. *Malaria Journal,* 7**,** 156.

Jones, C. M., Liyanapathirana, M., Agossa, F. R., Weetman, D., Ranson, H., Donnelly, M. J. & Wilding, C. S. 2012a. Footprints of positive selection associated with a mutation (N1575Y) in the voltage-gated sodium channel of *Anopheles gambiae*. *Proceedings of the National Academy of Sciences,* 109**,** 6614-6619.

Jones, C. M., Sanou, A., Guelbeogo, W. M., Sagnon, N., Johnson, P. & Ranson, H. 2012b. Aging partially restores the efficacy of malaria vector control in insecticide-resistant populations of *Anopheles gambiae* sl from Burkina Faso. *Malaria Journal,* 11**,** 10.1186.

Kabula, B., Kisinza, W., Tungu, P., Ndege, C., Batengana, B., Kollo, D., Malima, R., Kafuko, J., Mohamed, M. & Magesa, S. 2014. Co-occurrence and distribution of East (L1014S) and West (L1014F) African knock-down resistance in *Anopheles gambiae sensu lato* population of Tanzania. *Tropical Medicine & International Health,* 19**,** 331-341.

Kalinowski, S. 2009. How well do evolutionary trees describe genetic relationships among populations? *Heredity,* 102**,** 506-513.

Kamau, L., Lehmann, T., Hawley, W. A., Orago, A. S. S. & Collins, F. H. 1998. Microgeographic genetic differentiation of *Anopheles gambiae* mosquitoes from Asembo Bay, Western Kenya: A comparison with Kilifi in coastal Kenya. *American Journal of Tropical Medicine and Hygiene,* 58**,** 64-69.

Kamau, L., Mukabana, W. R., Hawley, W. A., Lehmann, T., Irungu, L. W., Orago, A. a. S. & Collins, F. H. 1999. Analysis of genetic variability in *Anopheles arabiensis* and *Anopheles gambiae* using microsatellite loci. *Insect Molecular Biology,* 8**,** 287-297.

Kamya, M. R., Arinaitwe, E., Wanzira, H., Katureebe, A., Barusya, C., Kigozi, S. P., Kilama, M., Tatem, A. J., Rosenthal, P. J. & Drakeley, C. 2015. Malaria transmission, infection, and disease at three sites with varied transmission intensity in Uganda: implications for malaria control. *The American Journal of Tropical Medicine and Hygiene,* 92**,** 903-912.

Kassen, R. 2002. The experimental evolution of specialists, generalists, and the maintenance of diversity. *Journal of Evolutionary Biology,* 15**,** pp. 173-190.

Keiser, J., Utzinger, J., De Castro, M. C., Smith, T. A., Tanner, M. & Singer, B. H. 2004. Urbanization in sub-Saharan Africa and implication for malaria control. *American Journal of Tropical Medicine and Hygiene,* 71**,** 118-127.

Kent, R. J. & Norris, D. E. 2005. Identification of mammalian blood meals in mosquitoes by a multiplexed polymerase chain reaction targeting cytochrome B. *The American Journal of Tropical Medicine and Hygiene,* 73**,** 336-342.

Khaemba, B. M., Mutani, A. & Bett, M. 1994. Studies of anopheline mosquitoes transmitting malaria in a newly developed highland urban area: a case study of Moi University and its environs. *East African Medical Journal,* 71**,** 159-164.

Killeen, G. F., Mckenzie, F. E., Foy, B. D., Bøgh, C. & Beier, J. C. 2001. The availability of potential hosts as a determinant of feeding behaviours and malaria transmission by African mosquito populations. *Transactions of the Royal Society of Tropical Medicine And Hygiene,* 95**,** 469-476.

Kleinschmidt, I., Mnzava, A. P., Kafy, H. T., Mbogo, C., Bashir, A. I., Bigoga, J., Adechoubou, A., Raghavendra, K., Knox, T. B. & Malik, E. M. 2015. Design of a study to determine the impact of insecticide resistance on malaria vector control: a multi-country investigation. *Malaria Journal,* 14**,** 282.

Kleinschmidt, I., Sharp, B., Benavente, L. E., Schwabe, C., Torrez, M., Kuklinski, J., Morris, N., Raman, J. & Carter, J. 2006. Reduction in infection with *Plasmodium falciparum* one year after the introduction of malaria control interventions on Bioko Island, Equatorial Guinea. *American Journal of Tropical Medicine and Hygiene,* 74**,** 972-978.

Klinkenberg, E., Mccall, P., Wilson, M. D., Akoto, A. O., Amerasinghe, F. P., Bates, I., Verhoeff, F. H., Barnish, G. & Donnelly, M. J. 2006. Urban malaria and anaemia in children: a cross-sectional survey in two cities of Ghana. *Tropical Medicine & International Health,* 11**,** 578-588.

Klinkenberg, E., Mccall, P., Wilson, M. D., Amerasinghe, F. P. & Donnelly, M. J. 2008. Impact of urban agriculture on malaria vectors in Accra, Ghana. *Malaria Journal,* 7**,** 151.

Klinkenberg, E., Mccall, P. J., Hastings, I. M., Wilson, M. D., Amerasinghe, F. P. & Donnelly, M. J. 2005. High malaria prevalence and urban agriculture in Accra, Ghana. *Emerg Infect Dis,* 11**,** 1290-1293.

Kloke, R. G., Nhamahanga, E., Hunt, R. H. & Coetzee, M. 2011. Vectorial status and insecticide resistance of *Anopheles funestus* from a sugar estate in southern Mozambique. *Parasit Vectors,* 4**,** 16.

Knols, B., Takken, W., Charlwood, D. & Jong, D. R. 1995. Species-specific attraction of *Anopheles* mosquitoes (Diptera: Culicidae) to different humans in South-East Tanzania.

Koekemoer, L., Kamau, L., Hunt, R. & Coetzee, M. 2002. A cocktail polymerase chain reaction assay to identify members of the *Anopheles funestus* (Diptera: Culicidae) group. *The American Journal of Tropical Medicine and Hygiene,* 66**,** 804-811.

Koekemoer, L., Waniwa, K., Brooke, B., Nkosi, G., Mabuza, A. & Coetzee, M. 2014. Larval salinity tolerance of two members of the *Anopheles funestus* group. *Medical and Veterinary Entomology,* 28**,** 187-192.

Koella, J. C. 1991. On the use of mathematical models of malaria transmission. *Acta Tropica,* 49**,** 1-25.

Koella, J. C. & Packer, M. J. 1996. Malaria parasites enhance blood-feeding of their naturally infected vector *Anopheles punctulatus*. *Parasitology,* 113**,** 105-109.

Koella, J. C., Sörensen, F. L. & Anderson, R. 1998. The malaria parasite, *Plasmodium falciparum,* increases the frequency of multiple feeding of its mosquito vector, *Anopheles gambiae*. *Proceedings of the Royal Society of London B: Biological Sciences,* 265**,** 763-768.

Kreuels, B., Kobbe, R., Adjei, S., Kreuzberg, C., Von Reden, C., Bäter, K., Klug, S., Busch, W., Adjei, O. & May, J. 2008. Spatial variation of malaria incidence in young children from a geographically homogeneous area with high endemicity. *Journal of Infectious Diseases,* 197**,** 85-93.

Kwiatkowska, R. M., Platt, N., Poupardin, R., Irving, H., Dabire, R. K., Mitchell, S., Jones, C. M., Diabaté, A., Ranson, H. & Wondji, C. S. 2013. Dissecting the mechanisms responsible for the multiple insecticide resistance phenotype in *Anopheles gambiae ss* M form, from Vallée de Kou, Burkina Faso. *Gene,* 519**,** 98-106.

Lansang, M. a. D., Belizario, V., Bustos, M., Saul, A. & Aguirre, A. 1997. Risk factors for infection with malaria in a low endemic community in Bataan, the Philippines. *Acta Tropica,* 63**,** 257-265.

Lefevre, T., Gouagna, L. C., Dabire, K. R., Elguero, E., Fontenille, D., Renaud, F., Costantini, C. & Thomas, F. 2010. Beer consumption increases human attractiveness to malaria mosquitoes. *PLoS One,* 5**,** e9546.

Lefevre, T., Gougna, L. C., Dabire, K. R., Elguero, E., Fontenille, D., Renaud, F., Constantini, C. & Thomas, F. 2009. Beyond Nature and Nurture: Phenotypic plasticity in blood-feeding behavior of *Anopheles gambiae* s.s. when humans are not readily accessible. *The American Journal of Tropical Medicine and Hygiene,* 81**,** pp. 1023-1029.

Lehmann, T., Hawley, W. A., Grebert, H., Danga, M., Atieli, F. & Collins, F. H. 1999. The rift valley complex as a barrier to gene flow for *Anopheles gambiae* in Kenya. *Journal of Heredity,* 90**,** 613-621.

Lehmann, T., Licht, M., Elissa, N., Maega, B. T. A., Chimumbwa, J. M., Watsenga, F. T., Wondji, C. S., Simard, F. & Hawley, W. A. 2003. Population structure of *Anopheles gambiae* in Africa. *Journal of Heredity,* 94**,** 133-147.

Lindblade, K. A., Mwandama, D., Mzilahowa, T., Steinhardt, L., Gimnig, J., Shah, M., Bauleni, A., Wong, J., Wiegand, R. & Howell, P. 2015. A cohort study of the

effectiveness of insecticide-treated bed nets to prevent malaria in an area of moderate pyrethroid resistance, Malawi. *Malaria Journal,* 14**,** 1-15.

Lindblade, K. A., Walker, E. D., Onapa, A. W., Katungu, J. & Wilson, M. L. 1999. Highland malaria in Uganda: prospective analysis of an epidemic associated with El Niño. *Transactions of the Royal Society of Tropical Medicine And Hygiene,* 93**,** 480-487.

Lindsay, S., Alonso, P., Schellenberg, J. A., Hemingway, J., Adiamah, J., Shenton, F., Jawara, M. & Greenwood, B. 1993a. A malaria control trial using insecticide-treated bed nets and targeted chemoprophylaxis in a rural area of The Gambia, West Africa 7. Impact of permethrin-impregnated bed nets on malaria vectors. *Transactions of the Royal Society of Tropical Medicine And Hygiene,* 87**,** 45-51.

Lindsay, S., Ansell, J., Selman, C., Cox, V., Hamilton, K. & Walraven, G. 2000. Effect of pregnancy on exposure to malaria mosquitoes. *The Lancet,* 355**,** 1972.

Lindsay, S., Schellenberg, J. A., Zeiler, H., Daly, R., Salum, F. & Wilkins, H. 1995. Exposure of Gambian children to *Anopheles gambiae* malaria vectors in an irrigated rice production area. *Medical and Veterinary Entomology,* 9**,** 50-58.

Lindsay, S. W., Adiamah, J. H., Miller, J. E., Pleass, R. J. & Armstrong, J. R. M. 1993b. Variation In Attractiveness Of Human-Subjects To Malaria Mosquitos (Diptera, Culicidae) In The Gambia. *Journal of Medical Entomology,* 30**,** 368-373.

Lindsay, S. W., Campbell, H., Adiamah, J. H., Greenwood, A. M., Bangali, J. E. & Greenwood, B. M. 1990. Malaria in a periurban area of The Gambia. *Annals of Tropical Medicine And Parasitology,* 84**,** 553-562.

Lindsay, S. W., Jawara, M., Paine, K., Pinder, M., Walraven, G. E. L. & Emerson, P. M. 2003. Changes in house design reduce exposure to malaria mosquitoes. *Tropical Medicine & International Health,* 8**,** 512-517.

Lindsay, S. W., Snow, R. W., Broomfield, G. L., Janneh, M. S., Wirtz, R. A. & Greenwood, B. M. 1989. Impact of permethrine treated bednets on malaria transmission by the *Anopheles gambiae* complex in The Gambia. *Medical and Veterinary Entomology,* 3**,** 263-271.

Lines, J. D. & Nassor, N. 1991. DDT resistance in *Anopheles gambiae* declines with mosquito age. *Medical and Veterinary Entomology,* 5**,** 261-265.

Lochouarn, L., Dia, I., Boccolini, D., Coluzzi, M. & Fontenille, D. 1998. Bionomical and cytogenetic heterogeneities of *Anopheles funestus* in Senegal. *Transactions of the Royal Society of Tropical Medicine And Hygiene,* 92**,** 607-612.

Lund, A. E. & Narahashi, T. 1983. Kinetics of sodium channel modification as the basis for the variation in the nerve membrane effects of pyrethroids and DDT analogs. *Pesticide Biochemistry And Physiology,* 20**,** 203-216.

Lynch, M. 2010. Evolution of the mutation rate. *Trends in Genetics,* 26**,** 345-352

Lynd, A., Weetman, D., Barbosa, S., Yawson, A. E., Mitchell, S., Pinto, J., Hastings, I. & Donnelly, M. J. 2010. Field, genetic, and modeling approaches show strong positive selection acting upon an insecticide resistance mutation in *Anopheles gambiae* ss. *Molecular Biology and Evolution,* 27**,** 1117-1125.

Macdonald, G. 1950. The analysis of infection rates in diseases in which super infection occurs. *Tropical Diseases Bulletin,* 47**,** 907-915.

Macdonald, G. 1953. The analysis of malaria epidemics. *Tropical Diseases Bulletin,* 50**,** 871.

Macdonald, G. 1956. Theory of the eradication of malaria. *Bulletin of the World Health Organization,* 15**,** 369.

Macdonald, G. 1965. Presidential address: On the scientific basis of tropical hygiene. *Transactions of the Royal Society of Tropical Medicine And Hygiene,* 59**,** 611-620.

Majambere, S., Fillinger, U., Sayer, D. R., Green, C. & Lindsay, S. W. 2008. Spatial distribution of mosquito larvae and the potential for targeted larval control in The Gambia. *The American Journal of Tropical Medicine and Hygiene,* 79**,** 19-27.

Majambere, S., Lindsay, S. W., Green, C., Kandeh, B. And Fillinger, U. 2007. Microbial larvicides for malaria control in The Gambia. *Malaria Journal,* 6.

Mala, A. O., Irungu, L. W., Shililu, J. I., Muturi, E. J., Mbogo, C. M., Njagi, J. K., Mukabana, W. R. & Githure, J. I. 2011. *Plasmodium falciparum* transmission and aridity: a Kenyan experience from the dry lands of Baringo and its implications for *Anopheles arabiensis* control. *Malaria Journal,* 10**,** 121.

Marascuilo, L. A. 1966. Large-sample multiple comparisons. *Psychological Bulletin,* 65**,** 280.

Marinotti, O., Calvo, E., Nguyen, Q. K., Dissanayake, S., Ribeiro, J. M. C. & James, A. A. 2006. Genome-wide analysis of gene expression in adult *Anopheles gambiae*. *Insect Molecular Biology,* 15**,** 1-12.

Marsh, K., Otoo, L., Hayes, R., Carson, D. & Greenwood, B. 1989. Antibodies to blood stage antigens of *Plasmodium falciparum* in rural Gambians and their relation to protection against infection. *Transactions of the Royal Society of Tropical Medicine And Hygiene,* 83**,** 293-303.

Martinez-Torres, D., Chandre, F., Williamson, M. S., Darriet, F., Berge, J. B., Devonshire, A. L., Guillet, P., Pasteur, N. & Pauron, D. 1998. Molecular characterization of pyrethroid knockdown resistance (kdr) in the major malaria vector *Anopheles gambiae s.s. Insect Molecular Biology,* 7**,** 179-184.

Mathenge, E. M., Gimnig, J. E., Kolczaz, M., Ombok, M., Irungu, L. W. & Hawley, W. A. 2001. Effect of permethrin-impregnated nets on exiting behavior, blood feeding

success, and time of feeding of malaria mosquitoes (Diptera: Culicidae) in Western Kenya. *Journal of Medical Entomology,* 38**,** 531-536.

Mathias, D. K., Ochomo, E., Atieli, F., Ombok, M., Bayoh, M. N., Olang, G., Muhia, D., Kamau, L., Vulule, J. M. & Hamel, M. J. 2011. Spatial and temporal variation in the kdr allele L1014S in *Anopheles gambiae* ss and phenotypic variability in susceptibility to insecticides in Western Kenya. *Malaria Journal,* 10**,** 10.

Mawejje, H. D., Wilding, C. S., Rippon, E. J., Hughes, A., Weetman, D. & Donnelly, M. J. 2013. Insecticide resistance monitoring of field-collected *Anopheles gambiae* s.l. populations from Jinja, eastern Uganda, identifies high levels of pyrethroid resistance. *Medical and Veterinary Entomology,* 27**,** 276-283.

Mbogo, C. M., Mwangangi, J. M., Nzovu, J., Gu, W., Yan, G., Gunter, J. T., Swalm, C., Keating, J., Regens, J. L. & Shililu, J. I. 2003. Spatial and temporal heterogeneity of *Anopheles* mosquitoes and *Plasmodium falciparum* transmission along the Kenyan coast. *The American Journal of Tropical Medicine and Hygiene,* 68**,** 734-742.

Mbogo, C. N. M., Baya, N. M., Ofulla, A. V. O., Githure, J. I. & Snow, R. W. 1996. The impact of permethrin-impregnated bednets on malaria vectors of the Kenyan coast. *Medical and Veterinary Entomology,* 10**,** 251-259.

Mccarroll, L. & Hemingway, J. 2002. Can insecticide resistance status affect parasite transmission in mosquitoes? *Insect Biochemistry and Molecular Biology,* 32**,** 1345-1351.

Mccarroll, L., Paton, M., Karunaratne, S., Jayasuryia, H., Kalpage, K. & Hemingway, J. 2000. Insecticides and mosquito-borne disease: insecticide resistance in mosquitoes can also interfere with developing parasites. *Nature (London),* 407**,** 961-962.

Mcgregor, I. A. & Smith, D. A. 1952. A health, nutrition and parasitological survey in a rural village (Keneba) in west Kiang, Gambia. *Transactions of the Royal Society of Tropical Medicine And Hygiene,* 46**,** 403-427.

Meirmans, P. G. 2006. Using the AMOVA framework to estimate a standardized genetic differentiation measure. *Evolution,* 60**,** 2399-2402.

Midega, J. T., Muturi, E. J., Baliraine, F. N., Mbogo, C. M., Githure, J., Beier, J. C. & Yan, G. 2010. Population structure of *Anopheles gambiae* along the Kenyan coast. *Acta Tropica,* 114**,** 103-108.

Midega, J. T., Smith, D. L., Olotu, A., Mwangangi, J. M., Nzovu, J. G., Wambua, J., Nyangweso, G., Mbogo, C. M., Christophides, G. K. & Marsh, K. 2012. Wind direction and proximity to larval sites determines malaria risk in Kilifi District in Kenya. *Nature Communications,* 3**,** 674.

Minakawa, N., Seda, P. & Yan, G. 2002. Influence of host and larval habitat distribution on the abundance of African malaria vectors in western Kenya. *The American Journal of Tropical Medicine and Hygiene,* 67**,** 32-38.

Mitchell, S. N., Rigden, D. J., Dowd, A. J., Lu, F., Wilding, C. S., Weetman, D., Dadzie, S., Jenkins, A. M., Regna, K. & Boko, P. 2014. Metabolic and target-site mechanisms combine to confer strong DDT resistance in *Anopheles gambiae*. *PLoS One,* 9**,** 92662.

Mitchell, S. N., Stevenson, B. J., Müller, P., Wilding, C. S., Egyir-Yawson, A., Field, S. G., Hemingway, J., Paine, M. J., Ranson, H. & Donnelly, M. J. 2012. Identification and validation of a gene causing cross-resistance between insecticide classes in *Anopheles gambiae* from Ghana. *Proceedings of the National Academy of Sciences,* 109**,** 6147-6152.

Moh 2012. National Health Policy 2012 - 2020. Ministry of Health and Social Welfare (ed.). Banjul, Gambia.

Moiroux, N., Gomez, M. B., Pennetier, C., Elanga, E., Djènontin, A., Chandre, F., Djègbé, I., Guis, H. & Corbel, V. 2012. Changes in *Anopheles funestus* biting behavior following universal coverage of long-lasting insecticidal nets in Benin. *Journal of Infectious Diseases,* 206**,** 1622-1629.

Molineaux, L., Shidrawi, G., Clarke, J., Boulzaguet, J. & Ashkar, T. 1979. Assessment of insecticidal impact on the malaria mosquito's vectorial capacity, from data on the man-biting rate and age-composition. *Bulletin of the World Health Organization,* 57**,** 265.

Mukabana, W. R., Takken, W., Coe, R. & Knols, B. G. J. 2002. Host-specific cues cause differential attractiveness of Kenyan men to the African malaria vector *Anopheles gambiae*. *Malaria Journal,* 1**,** 17.

Muller, P., Chouaibou, M., Pignatelli, P., Etang, J., Walker, E. D., Donnelly, M. J., Simard, F. & Ranson, H. 2008. Pyrethroid tolerance is associated with elevated expression of antioxidants and agricultural practice in *Anopheles arabiensis* sampled from an area of cotton fields in Northern Cameroon. *Molecular Ecology,* 17**,** 1145-1155.

Müller, P., Warr, E., Stevenson, B. J., Pignatelli, P. M., Morgan, J. C., Steven, A., Yawson, A. E., Mitchell, S. N., Ranson, H. & Hemingway, J. 2008. Field-caught permethrin-resistant *Anopheles gambiae* overexpress CYP6P3, a P450 that metabolises pyrethroids. *Plos Genetics,* 4**,** e1000286.

Mwangi, T. W., Fegan, G., Williams, T. N., Kinyanjui, S. M., Snow, R. W. & Marsh, K. 2008. Evidence for over-dispersion in the distribution of clinical malaria episodes in children. *PLoS One,* 3**,** e2196.

Mwesigwa, J., Okebe, J., Affara, M., Di Tanna, G. L., Nwakanma, D., Janha, O., Opondo, K., Grietens, K. P., Achan, J. & D'alessandro, U. 2015. On-going malaria transmission in The Gambia despite high coverage of control interventions: a nationwide cross-sectional survey. *Malaria Journal,* 14**,** 314.

N'guessan, R., Corbel, V., Akogbeto, M. & Rowland, M. 2007. Reduced efficacy of insecticide-treated nets and indoor residual spraying for malaria control in pyrethroid resistance area, Benin. *Emerging Infectious Diseases,* 13**,** 199-206.

Nájera, J. A. 1999. Malaria control: achievements, problems and strategies. Geneva: World Health Organization.

Narahashi, T. 2002. Nerve membrane ion channels as the target site of insecticides. *Mini reviews in medicinal chemistry,* 2**,** 419-432.

Ndiath, M. O., Cailleau, A., Diedhiou, S. M., Gaye, A., Boudin, C., Richard, V. & Trape, J.-F. 2014. Effects of the kdr resistance mutation on the susceptibility of wild *Anopheles gambiae* populations to *Plasmodium falciparum*: a hindrance for vector control. *Malaria Journal,* 13**,** 340.

Ndiath, M. O., Mazenot, C., Sokhna, C., Trape, J.F 2014. How the malaria vector *Anopheles gambiae* adapts to the use of insecticide-treated nets by African populations. *PLoS One,* 9**,** e97700.

Nei, M. 1987. *Molecular Evolutionary Genetics,* New York, Columbia University Press.

Newcombe, R. G. 1998a. Interval estimation for the difference between independent proportions: comparison of eleven methods. *Statistics in Medicine,* 17**,** 873-890.

Newcombe, R. G. 1998b. Two-sided confidence intervals for the single proportion: comparison of seven methods. *Statistics in Medicine,* 17**,** 857-872.

Ng'habi, K. R., Knols, B. G., Lee, Y., Ferguson, H. M. & Lanzaro, G. C. 2011. Population genetic structure of *Anopheles arabiensis* and *Anopheles gambiae* in a malaria endemic region of southern Tanzania. *Malaria Journal,* 10**,** 289.

Niang, E. H. A. 2014. *Variabilité génétique des populations d'Anopheles gambiae s.l. au Sénégal : implications dans le contrôle du paludisme.* Doctorat, Cheikh Anta Diop de Dakar.

Niang, E. H. A., Konaté, L., Diallo, M., Faye, O. & Dia, I. 2016. Patterns of insecticide resistance and knock down resistance (*kdr*) in malaria vectors *An. arabiensis*, *An. coluzzii* and *An. gambiae* from sympatric areas in Senegal. *Parasites & Vectors,* 9**,** 1.

Norris, L. C., Fornadel, C. M., Hung, W.-C., Pineda, F. J. & Norris, D. E. 2010. Frequency of multiple blood meals taken in a single gonotrophic cycle by *Anopheles arabiensis* mosquitoes in Macha, Zambia. *The American Journal of Tropical Medicine and Hygiene,* 83**,** 33-37.

Norris, L. C., Main, B. J., Lee, Y., Collier, T. C., Fofana, A., Cornel, A. J. & Lanzaro, G. C. 2015. Adaptive introgression in an African malaria mosquito coincident with the increased usage of insecticide-treated bed nets. *Proceedings of the National Academy of Sciences,* 112**,** 815-820.

Nwakanma, D. C., Neafsey, D. E., Jawara, M., Adiamoh, M., Lund, E., Rodrigues, A., Loua, K. M., Konate, L., Ngayo, S. Y., Dia, I., Awolola, T. S., Muskavitch, M. & Conway, D. J. 2013. Breakdown in the Process of Incipient Speciation in *Anopheles gambiae*. *Genetics,* 193**,** 1221-1231.

Obala, A. A., Kutima, H. L., Nyamogoba, H. D., Mwangi, A. W., Simiyu, C. J., Magak, G. N., Khwa-Otsyula, B. O. & Ouma, J. H. 2012. *Anopheles gambiae* and *Anopheles*

arabiensis population densities and infectivity in Kopere village, Western Kenya. *The Journal of Infection in Developing Countries,* 6**,** 637-643.

Ochomo, E., Bayoh, M., Brogdon, W., Gimnig, J., Ouma, C., Vulule, J. & Walker, E. 2013. Pyrethroid resistance in *Anopheles gambiae* ss and *Anopheles arabiensis* in western Kenya: phenotypic, metabolic and target site characterizations of three populations. *Medical and Veterinary Entomology,* 27**,** 156-164.

Ochomo, E., Bayoh, N. M., Kamau, L., Atieli, F., Vulule, J., Ouma, C., Ombok, M., Njagi, K., Soti, D. & Mathenge, E. 2014. Pyrethroid susceptibility of malaria vectors in four Districts of western Kenya. *Parasites and Vectors,* 7**,** 310-318.

Oesterholt, M., Bousema, J., Mwerinde, O., Harris, C., Lushino, P., Masokoto, A., Mwerinde, H., Mosha, F. & Drakeley, C. 2006. Spatial and temporal variation in malaria transmission in a low endemicity area in northern Tanzania. *Malaria Journal,* 5**,** 98.

Ogoma, S. B., Lorenz, L. M., Ngonyani, H., Sangusangu, R., Kitumbukile, M., Kilalangongono, M., Simfukwe, E. T., Mseka, A., Mbeyela, E. & Roman, D. 2014. An experimental hut study to quantify the effect of DDT and airborne pyrethroids on entomological parameters of malaria transmission. *Malaria Journal,* 13**,** 1.

Ogoma, S. B., Lweitoijera, D. W., Ngonyani, H., Furer, B., Russell, T. L., Mukabana, W. R., Killeen, G. F. & Moore, S. J. 2010. Screening mosquito house entry points as a potential method for integrated control of endophagic filariasis, arbovirus and malaria vectors. *PLoS Neglected Tropical Diseases,* 4**,** e773.

Okebe, J., Affara, M., Correa, S., Muhammad, A. K., Nwakanma, D., Drakeley, C. & D'alessandro, U. 2014. School-Based Countrywide Seroprevalence Survey Reveals Spatial Heterogeneity in Malaria Transmission in the Gambia. *PLoS One,* 9**,** e110926.

Oliveira, E., Salgueiro, P., Palsson, K., Vicente, J., Arez, A., Jaenson, T., Caccone, A. & Pinto, J. 2008. High levels of hybridization between molecular forms of *Anopheles gambiae* from Guinea Bissau. *Journal of Medical Entomology,* 45**,** 1057-1063.

Oliver, S. V. & Brooke, B. D. 2014. The effect of multiple blood-feeding on the longevity and insecticide resistant phenotype in the major malaria vector *Anopheles arabiensis* (Diptera: Culicidae). *Parasites and Vectors,* 7**,** 390.

Opondo, K. O., Weetman, D., Jawara, M., Diatta, M., Fofana, A., Crombe, F., Mwesigwa, J., D'alessandro, U. & Donnelly, M. J. 2016. Does insecticide resistance contribute to heterogeneities in malaria transmission in The Gambia? *Malaria Journal,* 15**,** 1.

Paaijmans, K. P., Read, A. F. & Thomas, M. B. 2009. Understanding the link between malaria risk and climate. *Proceedings of the National Academy of Sciences,* 106**,** 13844-13849.

Padonou, G., Sezonlin, M., Gbedjissi, G., Ayi, I., Azondekon, R., Djenontin, A., Bio-Bangana, S., Oussou, O., Yadouleton, A. & Boakye, D. 2011. Biology of *Anopheles gambiae* and insecticide resistance: Entomological study for a large scale of indoor residual spraying in South East Benin. *Journal of Parasitology and Vector Biology,* 3**,** 59-68.

Paetkau, D., Calvert, W., Stirling, I. & Strobeck, C. 1995. Microsatellite analysis of population structure in Canadian polar bears. *Molecular Ecology,* 4**,** 347-354.

Paetkau, D., Slade, R., Burden, M. & Estoup, A. 2004. Genetic assignment methods for the direct, real-time estimation of migration rate: a simulation-based exploration of accuracy and power. *Molecular Ecology,* 13**,** 55-65.

Page, R. D. M. 1996. TREEVIEW: An application to display phylogenetic trees on computers. *Computer Applications in the Biosciences,* 12**,** 357-358.

Pe, P. R. S. 2012. GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research—an update. *Bioinformatics,* 28**,** 2537-2539.

Peakall, R. & Smouse, P. E. 2006. GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes,* 6**,** 288-295.

Phillips, D. 1993. Urbanization and human health. *Parasitology,* 106**,** S93-S107.

Pinder, M., Jawara, M., Jarju, L. B., Kandeh, B., Jeffries, D., Lluberas, M. F., Mueller, J., Parker, D., Bojang, K., Conway, D. J. & Lindsay, S. 2011. To assess whether indoor residual spraying can provide additional protection against clinical malaria over current best practice of long-lasting insecticidal mosquito nets in The Gambia: study protocol for a two-armed cluster-randomised trial. *Trials,* 12**,** 147.

Pinder, M., Jawara, M., Jarju, L. B., Salami, K., Jeffries, D., Adiamoh, M., Bojang, K., Correa, S., Kandeh, B., Kaur, H., Conway, D. J., D'alessandro, U. & Lindsay, S. 2014. Efficacy of indoor residual spraying with dichlorodiphenyltrichloroethane against malaria in Gambian communities with high usage of long-lasting insecticidal mosquito nets: a cluster-randomised controlled trial. *The Lancet,* 385, 1436-1446.

Pinto, J., Lynd, A., Vicente, J. L., Santolamazza, F., Randle, N. P., Gentile, G., Moreno, M., Simard, F., Charlwood, J. D. & Do Rosário, V. E. 2007. Multiple origins of knockdown resistance mutations in the Afrotropical mosquito vector *Anopheles gambiae*. *PLoS One,* 2**,** e1243.

Piry, S., Alapetite, A., Cornuet, J.-M., Paetkau, D., Baudouin, L. & Estoup, A. 2004. GENECLASS2: a software for genetic assignment and first-generation migrant detection. *Journal of Heredity,* 95**,** 536-539.

Price, R. N., Tjitra, E., Guerra, C. A., Yeung, S., White, N. J. & Anstey, N. M. 2007. *Vivax* malaria: neglected and not benign. *The American Journal of Tropical Medicine and Hygiene,* 77**,** 79-87.

Pritchard, J. K., Stephens, M. & Donnelly, P. 2000. Inference of population structure using multilocus genotype data. *Genetics,* 155**,** 945-959.

Quiñones, M., Drakeley, C., Müller, O., Lines, J., Haywood, M. & Greenwood, B. 2000. Diversion of *Anopheles gambiae* from children to other hosts following exposure to permethrin-treated bednets. *Medical and Veterinary Entomology,* 14**,** 369-375.

Quiñones, M. L., Lines, J., Thomson, M. C., Jawara, M. & Greenwood, B. M. 1998. Permethrin-treated bed nets do not have a 'mass-killing effect'on village populations of *Anopheles gambiae* sl in The Gambia. *Transactions of the Royal Society of Tropical Medicine And Hygiene,* 92**,** 373-378.

Rajatileka, S., Burhani, J. & Ranson, H. 2011. Mosquito age and susceptibility to insecticides. *Transactions of the Royal Society of Tropical Medicine And Hygiene,* 105**,** 247-253.

Ranson, H., Paton, M. G., Jensen, B., Mccarroll, L., Vaughan, A., Hogan, J. R., Hemingway, J. & Collins, F. H. 2004. Genetic mapping of genes conferring permethrin resistance in the malaria vector, *Anopheles gambiae*. *Insect Molecular Biology,* 13**,** 379-386.

Ranson, H., Jensen, B., Vulule, J. M., Wang, X., Hemingway, J. & Collins, F. H. 2000. Identification of a point mutation in the voltage-gated sodium channel gene of Kenyan *Anopheles gambiae* associated with resistance to DDT and pyrethroids. *Insect Molecular Biology,* 9**,** 491-497.

Ranson, H. & Lissenden, N. 2016. Insecticide resistance in African *Anopheles* mosquitoes: A worsening situation that needs urgent action to maintain malaria control. *Trends in Parasitology,* 32**,** 187-196.

Ranson, H., N'guessan, R., Lines, J., Moiroux, N., Nkuni, Z. & Corbel, V. 2011. Pyrethroid resistance in African anopheline mosquitoes: what are the implications for malaria control? *Trends in Parasitology,* 27**,** 91-98.

Reddy, M. R., Overgaard, H. J., Abaga, S., Reddy, V. P., Caccone, A., Kiszewski, A. E. & Slotman, M. A. 2011. Outdoor host seeking behaviour of *Anopheles gambiae* mosquitoes following initiation of malaria vector control on Bioko Island, Equatorial Guinea. *Malaria Journal,* 10**,** 184.

Reimer, L., Fondjo, E., Patchoke, S., Diallo, B., Lee, Y., Ng, A., Ndjemai, H. M., Atangana, J., Traore, S. F., Lanzaro, G. & Cornel, A. J. 2008. Relationship between *kdr* mutation and resistance to pyrethroid and DDT insecticides in natural populations of *Anopheles gambiae*. *Journal of Medical Entomology,* 45**,** 260-266.

Reimer, L., Tripet, F., Slotman, M., Spielman, A., Fondjo, E. & Lanzaro, G. 2005. An unusual distribution of the kdr gene among populations of *Anopheles gambiae* on the island of Bioko, Equatorial Guinea. *Insect Molecular Biology,* 14**,** 683-688.

Riehle, M. M., Markianos, K., Niaré, O., Xu, J., Li, J., Touré, A. M., Podiougou, B., Oduol, F., Diawara, S. & Diallo, M. 2006. Natural malaria infection in *Anopheles gambiae* is regulated by a single genomic control region. *Science,* 312**,** 577-579.

Riveron, J. M., Irving, H., Ndula, M., Barnes, K. G., Ibrahim, S. S., Paine, M. J. I. & Wondji, C. S. 2013. Directionally selected cytochrome P450 alleles are driving the spread of pyrethroid resistance in the major malaria vector *Anopheles funestus*. *Proceedings of the National Academy of Sciences,* 110**,** 252-257.

Riveron, J. M., Yunta, C., Ibrahim, S. S., Djouaka, R., Irving, H., Menze, B. D., Ismail, H. M., Hemingway, J., Ranson, H. & Albert, A. 2014. A single mutation in the GSTe2 gene allows tracking of metabolically based insecticide resistance in a major malaria vector. *Genome Biology,* 15**,** 27.

Robert, V., Macintyre, K., Keating, J., Trape, J. F., Duchemin, J. B., Warren, M. & Beier, J. C. 2003. Malaria transmission in urban sub-Saharan Africa. *American Journal of Tropical Medicine and Hygiene,* 68**,** 169-176.

Rousset, F. 2008. genepop'007: a complete re-implementation of the genepop software for Windows and Linux. *Molecular Ecology Resources,* 8**,** 103-106.

Rundle, H. D. & Nosil, P. 2005. Ecological speciation. *Ecology letters,* 8**,** 336-352.

Russell, T. L., Govella, N. J., Azizi, S., Drakeley, C., Kachur, P. & Killeen, G. F. 2011. Increased proportions of outdoor feeding among residual malaria vector populations following increased use of insecticide-treated nets in rural Tanzania. *Malaria Journal,* 10.

Russell, T. L., Lwetoijera, D. W., Maliti, D., Chipwaza, B., Kihonda, J., Charlwood, J. D., Smith, T. A., Lengeler, C., Mwanyangala, M. A., Nathan, R., Knols, B. G., Takken, W. & Killeen, G. F. 2010. Impact of promoting longer-lasting insecticide treatment of bed nets upon malaria transmission in a rural Tanzanian setting with pre-existing high coverage of untreated nets. *Malaria Journal,* 9**,** 187.

Saddler, A. & Koella, J. C. 2015. Modelling the impact of declining insecticide resistance with mosquito age on malaria transmission. *MalariaWorld Journal,* 6**,** 13.

Samcp 2001. Kwazulu/Natal Malaria Crisis Progress Report. South Africa: KwaZulu/Natal Department of Health Statistics.

Santolamazza, F., Calzetta, M., Etang, J., Barrese, E., Dia, I., Caccone, A., Donnelly, M. J., Petrarca, V., Simard, F., Pinto, J. & Della Torre, A. 2008a. Distribution of knock-down resistance mutations in *Anopheles gambiae* molecular forms in west and west-central Africa *Malaria Journal,* 7**,** e74.

Santolamazza, F., Caputo, B., Nwakanma, D. C., Fanello, C., Petrarca, V., Conway, D. J., Weetman, D., Pinto, J., Mancini, E. & Della Torre, A. 2015. Remarkable diversity of intron-1 of the para voltage-gated sodium channel gene in an *Anopheles gambiae*/*Anopheles coluzzii* hybrid zone. *Malaria Journal,* 14**,** 9.

Santolamazza, F., Mancini, E., Simard, F., Qi, Y., Tu, Z. & Della Torre, A. 2008b. Insertion polymorphisms of SINE200 retrotransposons within speciation islands of *Anopheles gambiae* molecular forms. *Malaria Journal,* 7**,** 163.

Sattler, M. A., Mtasiwa, D., Kiama, M., Premji, Z., Tanner, M., Killeen, G. F. & Lengeler, C. 2005. Habitat characterization and spatial distribution of *Anopheles sp.* mosquito larvae in Dar es Salaam (Tanzania) during an extended dry period. *Malaria Journal,* 4**,** e4.

Schluter, D. 2000. *The Ecology of Adaptive Radiation,* OUP Oxford.

Scott, J. A., Brogdon, W. G. & Collins, F. H. 1993. Identification of single specimens of the *Anopheles gambiae* complex by the Polymerase Chain Reaction. *American Journal of Tropical Medicine and Hygiene,* 49**,** 520-529.

Scott, T. W., Githeko, A. K., Fleisher, A., Harrington, L. C. & Yan, G. 2006. DNA profiling of human blood in anophelines from lowland and highland sites in western Kenya. *The American Journal of Tropical Medicine and Hygiene,* 75**,** 231-237.

Service, M. W. 1977. A critical review of procedures for sampling populations of adult mosquitoes. *Bulletin of Entomological Research,* 67**,** pp. 343-382.

Service, M. W. 1982. Importance of vector ecology in vector disease control in Africa. *Bulletin of Society of Vector Ecology,* 7**,** pp.1-13.

Service, M. W. 1997. Mosquito (Diptera : Culicidae) dispersal - The long and short of it. *Journal Of Medical Entomology,* 34**,** 579-588.

Seyoum, A., Sikaala, C. H., Chanda, J., Chinula, D., Ntamatungiro, A. J., Hawela, M., Miller, J. M., Russell, T. L., Briët, O. J. T. & Killeen, G. F. 2012. Human exposure to anopheline mosquitoes occurs primarily indoors, even for users of insecticide-treated nets in Luangwa Valley, South-east Zambia. *Parasites and Vectors,* 5**,** 101.

Shafer, A. & Wolf, J. B. 2013. Widespread evidence for incipient ecological speciation: a meta-analysis of isolation-by-ecology. *Ecology Letters,* 16**,** 940-950.

Sharp, B. L., Ridl, F. C., Govender, D., Kuklinski, J. & Kleinschmidt, I. 2007. Malaria vector control by indoor residual insecticide spraying on the tropical island of Bioko, Equatorial Guinea. *Malaria Journal,* 6**,** 52.

Sikulu, M., Killeen, G. F., Hugo, L. E., Ryan, P. A., Dowell, K. M., Wirtz, R. A., Moore, S. J. & Dowell, F. E. 2010. Near-infrared spectroscopy as a complementary age grading and species identification tool for African malaria vectors. *Parasites and Vectors,* 3**,** 1.

Slatkin, M. 1993. Isolation by distance in equilibrium and non-equilibrium populations. *Evolution***,** 264-279.

Smith, D. L., Dushoff, J. & Mckenzie, F. E. 2004. The risk of a mosquito-Borne infection in a heterogenous environment. *PLoS Biology,* 2**,** e368.

Smith, T., Charlwood, J., Takken, W., Tanner, M. & Spiegelhalter, D. 1995. Mapping the densities of malaria vectors within a single village. *Acta Tropica,* 59**,** 1-18.

Smith, T., Charlwood, J. D., Kihonda, J., Mwankusye, S., Billingsley, P., Meuwissen, J., Lyimo, E., Takken, W., Teuscher, T. & Tanner, M. 1993. Absence of seasonal variation in malaria parasitaemia in an area of intense seasonal transmission. *Acta Tropica,* 54**,** 55-72.

SNMCP 2011. Profile Entomologique du paludisme au Senegal. Ministere de la Sante et de la prevention Medicale Profil.

Soper, F. L. & Wilson, B. D. 1943. *Anopheles gambiae in Brazil, 1930 to 1940,* New York, Rockerfeller Foundation.

Soper, F. L. & Wilson, D. B. 1942. Species eradication. A practical goal of species reduction in the control of mosquito-borne disease. *Journal of the National Malaria Society,* 1**,** 5-24.

Sougoufara, S., Diédhiou, S. M., Doucouré, S., Diagne, N., Sembène, P. M., Harry, M., Trape, J.-F., Sokhna, C. & Ndiath, M. O. 2014. Biting by *Anopheles funestus* in broad daylight after use of long-lasting insecticidal nets: a new challenge to malaria elimination. *Malaria Journal,* 13**,** 10.1186.

Sparks, T. C., Lockwood, J. A., Byford, R. L., Graves, J. B. & Leonard, B. R. 1989. The role of behavior in insecticide resistance. *Pesticide Science,* 26**,** 383-399.

Stoddard, S. T., Morrison, A. C., Vazquez-Prokopec, G. M., Soldan, V. P., Kochel, T. J., Kitron, U., Elder, J. P. & Scott, T. W. 2009. The role of human movement in the transmission of vector-borne pathogens. *PLoS Neglected Tropical Diseases,* 3**,** e481.

Stump, A. D., Atieli, F. K., Vulule, J. M. & Besansky, N. J. 2004. Dynamics of the pyrethroid knockdown resistance allele in western Kenyan populations of *Anopheles gambiae* in response to insecticide-treated bed net trials. *American Journal of Tropical Medicine and Hygiene,* 70**,** 591-596.

Su, H., Qu, L., He, K., Zhang, Z., Wang, J., Chen, Z. & Gu, H. 2003. The Great Wall of China: a physical barrier to gene flow? *Heredity,* 90**,** 212-219.

Takem, E. N., Affara, M., Amambua-Ngwa, A., Okebe, J., Ceesay, S. J., Jawara, M., Oriero, E., Nwakanma, D., Pinder, M., Clifford, C., Taal, M., Sowe, M., Suso, P., Mendy, A., Mbaye, A., Drakeley, C. & D'alessandro, U. 2013. Detecting foci of malaria transmission with school surveys: a pilot study in the Gambia. *PLoS One,* 8**,** e67108.

Tangena, J., Adiamoh, M., D'alessandro, U., Jarju, L., Jawara, M., Jeffries, D., Malik, N., Nwakanma, D., Kaur, H. & Takken, W. 2013. Alternative treatments for indoor residual spraying for malaria control in a village with pyrethroid-and DDT-resistant vectors in the Gambia. *PLoS One,* 8**,** e74351.

Tanner, M. & Savigny, D. D. 2008. Malaria eradication back on the table. *Bulletin of the World Health Organization,* 86**,** 82-82.

Taylor, B. 1975. Changes in the feeding behaviour of a malaria vector, *Anopheles farauti* Lav., following use of DDT as a residual spray in houses in the British Solomon Islands Protectorate. *Transactions of the Royal Entomological Society of London,* 127**,** 277-292.

Taylor, C., Touré, Y. T., Carnahan, J., Norris, D. E., Dolo, G., Traoré, S. F., Edillo, F. E. & Lanzaro, G. C. 2001. Gene flow among populations of the malaria vector, *Anopheles gambiae,* in Mali, West Africa. *Genetics,* 157**,** 743-750.

Thomas, C. & Lindsay, S. 2000. Local-scale variation in malaria infection amongst rural Gambian children estimated by satellite remote sensing. *Transactions of the Royal Society of Tropical Medicine And Hygiene,* 94**,** 159-163.

Thomas, C. J., Cross, D. E. & Bøgh, C. 2013. Landscape movements of *Anopheles gambiae* malaria vector mosquitoes in rural Gambia. *PLoS One,* 8**,** e68679

Thomson, M., D'alessandro, U., Bennett, S., Connor, S., Langerock, P., Jawara, M., Todd, J. & Greenwood, B. 1994. Malaria prevalence is inversely related to vector density in The Gambia, West Africa. *Transactions of the Royal Society of Tropical Medicine And Hygiene,* 88**,** 638-643.

Tokponnon, F. T., Ogouyémi, A. H., Sissinto, Y., Sovi, A., Gnanguenon, V., Cornélie, S., Adéothy, A. A., Ossè, R., Wakpo, A. & Gbénou, D. 2014. Impact of long-lasting, insecticidal nets on anaemia and prevalence of *Plasmodium falciparum* among children under five years in areas with highly resistant malaria vectors. *Malaria Journal,* 13**,** 76.

Trape, J.-F., Tall, A., Diagne, N., Ndiath, O., Ly, A. B., Faye, J., Dieye-Ba, F., Roucher, C., Bouganali, C. & Badiane, A. 2011. Malaria morbidity and pyrethroid resistance after the introduction of insecticide-treated bednets and artemisinin-based combination therapies: a longitudinal study. *The Lancet Infectious Diseases,* 11**,** 925-932.

Trape, J. F., Lefebvrezante, E., Legros, F., Ndiaye, G., Bouganali, H., Druilhe, P. & Salem, G. 1992. Vector density gradients and the epidemiology of urban malaria in Dakar, Senegal. *American Journal of Tropical Medicine and Hygiene,* 47**,** 181-189.

Trape, J. F., Pison, G., Spiegel, A., Enel, C. & Rogier, C. 2002. Combating malaria in Africa. *Trends in Parasitology,* 18**,** 224-230.

Un 2014. World Urbanization Prospects: the 2014 Revision. New York: United Nations, Department of Economic and Social affairs.

Van Oosterhout, C., Hutchinson, W. F., Wills, D. P. & Shipley, P. 2004. MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology Notes,* 4**,** 535-538.

Vezenegho, S. B., Chiphwanya, J., Hunt, R. H., Coetzee, M., Bass, C. & Koekemoer, L. L. 2013. Characterization of the *Anopheles funestus* group, including *Anopheles funestus*-like, from northern Malawi. *Transactions of the Royal Society of Tropical Medicine And Hygiene,* 107**,** 753-762.

Vontas J, David Jp, Nikou D, Hemingway J, Christophides Gk, Louis C & H., R. 2007. Transcriptional analysis of insecticide resistance in *Anopheles stephensi* using cross-species microarray hybridization. *Insect Molecular Biology,* 16**,** 315-324.

Wahlund, S. 1928. The combination of populations and the appearance of correlation examined from the standpoint of the study of heredity. *Hereditas,* 11**,** 65-106.

Waples, R. S. & Gaggiotti, O. 2006. Invited Review: What is a population? An empirical evaluation of some genetic methods for identifying the number of gene pools and their degree of connectivity. *Molecular Ecology,* 15**,** 1419-1439.

Weetman, D. & Donnelly, M. J. 2015. Evolution of insecticide resistance diagnostics in malaria vectors. *Transactions of the Royal Society of Tropical Medicine And Hygiene,* 109**,** 291-293.

Weetman, D., Wilding, C. S., Steen, K., Pinto, J. & Donnelly, M. J. 2012. Gene flow–dependent genomic divergence between *Anopheles gambiae* M and S forms. *Molecular Biology and Evolution,* 29**,** 279-291.

Weill, M., Chandre, F., Brengues, C., Manguin, S., Akogbeto, M., Pasteur, N., Guillet, P. & Raymond, M. 2000. The *kdr* mutation occurs in the Mopti form of *Anopheles gambiae s.s.* through introgression. *Insect Molecular Biology,* 9**,** 451-455.

Weill, M., Fort, P., Berthomieu, A., Dubois, M. P., Pasteur, N. & Raymond, M. 2002. A novel acetylcholinesterase gene in mosquitoes codes for the insecticide target and is

non–homologous to the ace gene *Drosophila*. *Proceedings of the Royal Society of London B: Biological Sciences,* 269**,** 2007-2016.

Weill, M., Malcolm, C., Chandre, F., Mogensen, K., Berthomieu, A., Marquine, M. & Raymond, M. 2004. The unique mutation in ace-1 giving high insecticide resistance is easily detectable in mosquito vectors. *Insect Molecular Biology,* 13**,** 1-7.

Weir, B. S. & Cockerham, C. C. 1984. Estimating F-statistics for the analysis of population structure. *Evolution,* 38**,** 1358-1370.

Wekesa, J. W., Copeland, R. S. & Mwangi, R. W. 1992. Effect of *Plasmodium falciparum* on blood feeding behavior of naturally infected *Anopheles* mosquitoes in western Kenya. *The American Journal of Tropical Medicine and Hygiene,* 47**,** 484-488.

Wesolowski, A., Eagle, N., Tatem, A. J., Smith, D. L., Noor, A. M., Snow, R. W. & Buckee, C. O. 2012. Quantifying the impact of human mobility on malaria. *Science,* 338**,** 267-270.

White, G. & Rosen, P. 1973. Comparative studies on sibling species of the *Anopheles gambiae* Giles complex (Dipt., Culicidae). II. Ecology of species A and B in savanna around Kaduna, Nigeria, during transition from wet to dry season. *Bulletin of Entomological Research,* 62**,** 613-625.

WHO 2007. Malaria elimination: a field manual for low and moderate endemic countries. Geneva: World Health Organization.

WHO 2012. Global Plan for Insecticide Resistance Management in Malaria Vectors. Geneva.

WHO 2013a. Test procedures for insecticide resistance monitoring in malaria vector mosquitoes. Geneva: World Health Organization.

WHO 2013b. World Malaria Report 2013. Geneva: World Health Organization.

WHO 2015a. Global Technical Strategy for Malaria 2016-2030. United Kingdom: World Health Organization.

WHO 2015b. World Malaria Report 2015. Geneva: World Health Organization.

Williamson, T., Eliasziw, M. & Fick, G. H. 2013. Log-binomial models: exploring failed convergence. *Emerging Themes in Epidemiology,* 10**,** 14.

Wilson, E. B. 1927. Probable inference, the law of succession, and statistical inference. *Journal of the American Statistical Association,* 22**,** 209-212.

Wondji, C. S., Coleman, M., Kleinschmidt, I., Mzilahowa, T., Irving, H., Ndula, M., Rehman, A., Morgan, J., Barnes, K. G. & Hemingway, J. 2012. Impact of pyrethroid resistance on operational malaria control in Malawi. *Proceedings of the National Academy of Sciences,* 109**,** 19063-19070.

Wondji, C. S., Irving, H., Morgan, J., Lobo, N. F., Collins, F. H., Hunt, R. H., Coetzee, M., Hemingway, J. & Ranson, H. 2009. Two duplicated P450 genes are associated with pyrethroid resistance in *Anopheles funestus*, a major malaria vector. *Genome Research,* 19**,** 452-459.

Woolhouse, M. E., Dye, C., Etard, J.-F., Smith, T., Charlwood, J., Garnett, G., Hagan, P., Hii, J., Ndhlovu, P. & Quinnell, R. 1997. Heterogeneities in the transmission of infectious agents: implications for the design of control programs. *Proceedings of the National Academy of Sciences,* 94**,** 338-342.

Yawson, A. E., Weetman, D., Wilson, M. D. & Donnelly, M. J. 2007. Ecological zones rather than molecular forms predict genetic differentiation in the malaria vector *Anopheles gambiae s.s.* in Ghana. *Genetics,* 175**,** 751-761.

Yohannes, M. & Boelee, E. 2012. Early biting rhythm in the afro-tropical vector of malaria, *Anopheles arabiensis,* and challenges for its control in Ethiopia. *Medical and Veterinary Entomology,* 26**,** 103-105.

Yue, J. C. & Clayton, M. K. 2005. A similarity measure based on species proportions. *Communications in Statistics-Theory and Methods,* 34**,** 2123-2131.

Yusuf, O., Bamgboye, E., Afolabi, R. & Shodimu, M. 2014. An appraisal of convergence failures in the application of logistic regression model in published manuscripts. *African Journal of Medicine and Medical Sciences,* 43**,** 195-204.

Zhou, G., Afrane, Y. A., Vardo-Zalik, A. M., Atieli, H., Zhong, D., Wamae, P., Himeidan, Y. E., Minakawa, N., Githeko, A. K. & Yan, G. 2011. Changing patterns of malaria epidemiology between 2002 and 2010 in Western Kenya: the fall and rise of malaria. *PLoS One,* 6**,** e20318.

Zhou, G., Munga, S., Minakawa, N., Githeko, A. K. & Yan, G. 2007. Spatial relationship between adult malaria vector abundance and environmental factors in western Kenya highlands. *The American Journal of Tropical Medicine and Hygiene,* 77**,** 29-35.

APPENDICES

Appendix 1 Published paper in Malaria journal

Opondo *et al. Malar J (2016) 15:166*
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Malaria Journal

RESEARCH Open Access

Does insecticide resistance contribute to heterogeneities in malaria transmission in The Gambia?

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Abstract

Background: Malaria hotspots, areas with consistently higher than average transmission, may become increasingly common as malaria declines. This phenomenon, currently observed in The Gambia, may be caused by several factors, including some related to the local vectors, whose contribution is poorly understood.

Methods: Using WHO susceptibility bioassays, insecticide resistance status was determined in vector populations sampled from six pairs of villages across The Gambia, each pair contained a low and high prevalence village.

Results: Three vector species were observed (23.5 % *Anopheles arabiensis*, 31.2 % *Anopheles gambiae*, 43.3 % *Anopheles coluzzii* and 2.0 % *An. coluzzii* × *An. gambiae* hybrids). Even at a fine scale, significant differences in species composition were detected within village pairs. Resistance to both DDT and deltamethrin was more common in *An. gambiae*, most markedly in the eastern part of The Gambia and partly attributable to differing frequencies of resistance mutations. The *Vgsc-1014F* target site mutation was strongly associated with both DDT (OR = 256.7, (95 % CI 48.6–6374.3, $p < 0.001$) and deltamethrin survival (OR = 9.14, (95 % CI 4.24–21.4, $p < 0.001$). A second target site mutation, *Vgsc-1575Y*, which co-occurs with *Vgsc-1014F*, and a metabolic marker of resistance, *Gste2-114T*, conferred additional survival benefits to both insecticides. DDT resistance occurred significantly more frequently in villages with high malaria prevalence ($p = 0.025$) though this did not apply to deltamethrin resistance.

Conclusion: Whilst causality of relationships requires further investigation, variation in vector species and insecticide resistance in The Gambia is associated with malaria endemicity; with a notably higher prevalence of infection and insecticide resistance in the east of the country. In areas with heterogeneous malaria transmission, the role of the vector should be investigated to guide malaria control interventions.

Background

Malaria foci, referred to as 'hot spots', have persistently higher transmission rates [1–3] than contiguous areas and pose challenges to malaria control programmes. They may be refractory to conventional malaria control tools and may act as sources of infection to surrounding areas [4, 5]. As transmission falls, partly in response to control scale-up, [6] heterogeneity in transmission will become more apparent [7, 8]. Marked heterogeneity in transmission has been documented [9, 10] even at the village level [11, 12], and in areas of overall reduced transmission like The Gambia [13, 14].

Understanding the epidemiological factors that contribute to the emergence and maintenance of these hotspots is crucial for malaria elimination. Human [15–17] and vector behaviour [18, 19], environmental factors [20–23] and their interplay may give an insight into the transmission dynamics in hotspots. Malaria vector species and populations vary in space and time [18], in anthropophily, exophily and endophily [19] and, importantly, in insecticide susceptibility [24].

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Resistance to available insecticides has been widely reported in malaria vectors [25–31]. Although a causal relationship between insecticide resistance and malaria transmission has not been shown, spatial variation in susceptibility to insecticides is likely to contribute to the observed heterogeneity in malaria transmission [32]. Since mosquitoes resistant to insecticides survive longer than their susceptible counterparts in the presence of an insecticide, they may live long enough [33] to affect malaria transmission [34–36]. Therefore, insecticide-resistant vectors may maintain transmission [37] or, where control interventions have been successful, reverse gains [35, 38, 39].

Malaria in The Gambia

In The Gambia, malaria transmission has decreased substantially over the last few years and has become increasingly heterogeneous [6, 13, 14]. Malaria transmission follows rainfall, beginning after the onset of the rains and peaking between October and November. Malaria prevalence in children under the age of 5 years is nationally 4–5 %, though in some areas between 2 and 15 % [6, 14, 40]. In the eastern Gambia, cross-sectional survey across all ages in 2012 estimated malaria prevalence at above 30 %.

Malaria control, coordinated by the Gambia National Malaria Control Programme (GNMCP), largely employs long-lasting insecticide-treated bed nets (LLINs) and indoor residual spraying (IRS) with DDT [41]. Between 2013 and 2014, the GNMCP carried out a mass LLIN distribution campaign with Permanet®. While a cross-sectional survey across Gambian villages showed over 90 % bed net use in 2012 [13], the National LLIN usage in children under the age of 5 years stands at 60 % while in pregnant women it is only 40 % [14]. Annual IRS with DDT has been done since 2008 throughout the country except the coastal region where malaria transmission is extremely low. The first-line treatment is artemether-lumefantrine; pregnant women receive sulfadoxine-pyrimethamine as intermittent preventive treatment while children 3–59 months old in upper and central river regions (URR and CRR) obtain seasonal malaria chemoprevention with amodiaquine and sulfadoxine-pyrimethamine since the 2014 transmission season.

Vector control activities carried out by GNMCP have probably played a major role in reducing transmission [14]. However, these gains may be reversed by insecticide resistance that has been recently observed in The Gambia [42, 43]. Vector species distribution varies from east to west along the River Gambia [44]. Four malaria vectors, *Anopheles gambiae s.s., Anopheles coluzzii, Anopheles arabiensis* and *Anopheles melas* maintain transmission. *Anopheles melas* is mainly confined to brackish waters near the coastal region but extends up to approximately 200 km inland during the rainy season [44–46]. During the rainy season, the population of *An. gambiae s.s.* rises non-uniformly across the country while *An. arabiensis* and *An. coluzzii* persist longer into the dry season [44].

The local dynamics of insecticide resistance may be impacted by the spatio-temporal variation in insect vectors [47–49], which can result from different ecological niche preferences [50, 51]. In scenarios where populations are separated by ecological factors or barriers, different resistance mechanisms may develop as a result of differential selection pressure or the occurrence of different mutations. Nonetheless, occasional gene flow [52] can transfer mutations [53–56] which may rise rapidly in frequency if selected by anthropogenic activity.

As part of a larger study investigating malaria transmission dynamics in The Gambia, the distribution and patterns of phenotypic resistance and mechanisms in *An. gambiae s.l.* populations was characterized. Specifically, the hypothesis that variation in the intensity of malaria transmission may be linked with variation in insecticide resistance, mediated by differences in species composition and resistance-related mutations was examined.

Methods

Study sites

The study was conducted in The Gambia, a West African country surrounded by Senegal except to the west that borders the Atlantic Ocean. The country is divided into five administrative regions, namely west coast, lower river region—south (LRR-south), lower river region—north (LRR-north), central river region (CRR) and upper river region (URR) (Fig. 1). For purposes of this study and the overall study investigating transmission dynamics, URR was subdivided into URR—north and south to form a total of six regions. Six pairs of rural villages, one pair per region, were selected on the basis of malaria prevalence determined by a nationwide cross-sectional survey [13] (Fig. 1). In each pair, the village with the highest prevalence and that with the lowest prevalence were included. For all pairs there was a significant difference in infection prevalence with the exception of villages G and H in the central river region (Additional file 1: Table S1).

The Gambia has one rainy season from June to October diminishing in November. The mean daily temperature varies between 25 and 40 °C. The country is primarily low lying with seasonal flooding; and is situated in the open and flat woodland Savannah belt and riverine swamps are common towards the western part of the country [44, 57]. The sea mixes with the river and during the rainy season, brackish waters can extend 200 km upstream. Rice paddies are common on the margins of the river, especially in the CRR. Towards the east, cereal crop

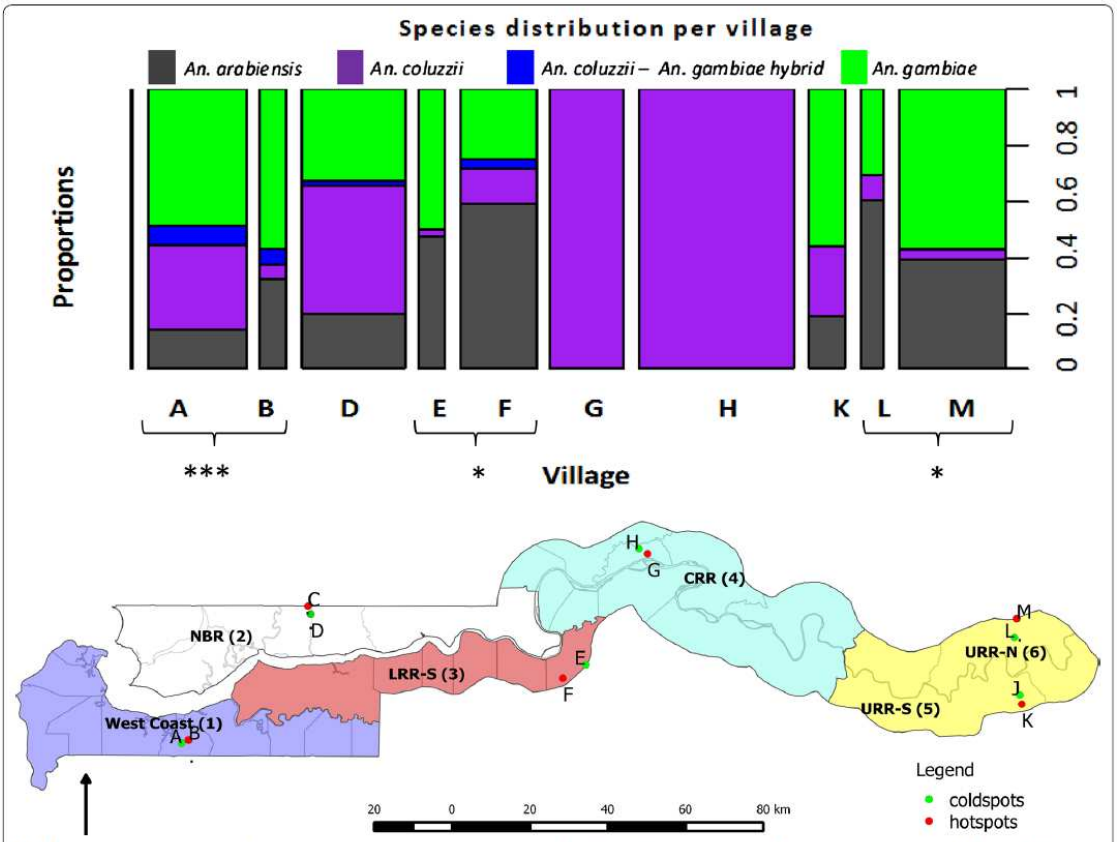

Fig. 1 Graph showing the relative proportion of malaria vectors across study sites in The Gambia. *Asterisks* indicated significant differences in species diversity between village pairs assessed using fisher exact tests (where collections and > 1 species collected, permitted test); ***P < 0.001; *P < 0.05 Below it is administrative map of The Gambia showing study sites/villages in 6 geographic regions (numbered). *1* west coast, *2* LRR-N (lower river region-north), *3* LRR-S (lower river region-south), *4* CRR-N (central river region-north), *5/6* URR-S/N (upper river region-north/south). The *green dots* indicate low malaria prevalence while *red dots*—high malaria prevalence villages. *A* Bessi, *B* Ndemban Tenda, *C* Chogen Wellingara, *D* Yallal Ba, *E* Sinchu Njengudi, *F* Dongoro Ba, *G* Sare Seedy, *H* Ngedden, *K* Madina Samako, *J* Njaiyel, *L* Sare Wuro, *M* Gunjur Koto

farming is practised. Between the months of June and September 2013, coinciding with mosquito sampling, the GNMCP distributed LLINs and sprayed houses with DDT in the country including all our study villages with the villages in region 2 being sprayed last in the months of September/October.

Study design

Mosquitoes were sampled between July and October 2013 from the 12 villages. Larval collections were conducted within a 2 km radius of the centre of the villages and transported to a central insectary in Wali Kunda (13°34′N, 14°55′W) for rearing and testing. Blood fed adult female collections were performed in villages that had few or no observable breeding habitats. Blood-fed anophelines were transferred to the insectary on the same day of collection where they were kept in individual paper cups containing moistened Whatman filter papers to induce egg laying. The females were also provided with 10 % glucose solution on a cotton wool plug. Eggs from blood-fed mosquitoes from one village were grouped together and allowed to mix randomly.

Mosquitoes, including an insecticide-susceptible colony from Yaoundé, Cameroon, were reared under similar conditions. Larvae were fed on Tetramin® (Tetramin gmbH Germany) fish food and maintained at 28 °C and 80 % humidity. Upon emergence, adult mosquitoes were provided with 10 % glucose. The WHO protocol [58] on insecticide susceptibility tube assays was used to assay phenotypic resistance.

Three to five day old mosquitoes in groups of 20–25 were exposed for an hour to either 4 % DDT or 0.05 % deltamethrin impregnated papers [58]. These two insecticides were chosen because the GNMCP distributes deltamethrin-impregnated LLINs (Permanet®) and uses DDT in IRS campaigns. A total of 1005 field collected *An. gambiae s.l.* were tested. Mortality in the control group (susceptible colony from Yaoundé Cameroon) was always less than 5 %. After the phenotypic assays, all mosquitoes tested were stored in 1.5 ml Eppendorf tubes with silica gel and transported to the MRC Fajara for species identification and molecular screening of insecticide resistance loci.

Laboratory processing

DNA from all mosquitoes was extracted using a Qiagen kit according to manufacturer's protocol. Two polymerase chain reaction protocols [59, 60] were used to identify the *An. gambiae s.l.* to species level. The protocol of Scott et al. was used to identify *An. gambiae s.s.*, *An. arabiensis*, *An. melas* while the SINE-PCR [60] protocol was used to further distinguish the *An. gambiae s.s.*, from *An. coluzzii* and *An. arabiensis*, simultaneously.

All mosquitoes tested in the insecticide resistance bioassay were genotyped, using TaqMan assays [61–64], for five markers of insecticide resistance, namely the *Vgsc-1014F* and *Vgsc-1014S* mutations in the voltage gated sodium channel gene that confer resistance to DDT/pyrethroids, *Vgsc-1575Y* which enhances action of the *1014F* mutation, *Gste2-114T* which has been associated with metabolic resistance to DDT, and *Ace1-119S* which is associated with resistance to carbamates and organophosphates [63].

Statistics

Statistical analysis was done using R statistical package (R version 3.1.2, 2014). Tests of differences in proportions were done to investigate differences in vector populations. Fisher's test was used to determine differences in species composition using an online algorithm. Pearson's Chi squared test for proportions was used to test for differences in mortality between species and villages. Non-parametric tests were used to investigate differences in mortality to insecticides within pairs of study villages and, more generally, geographic variation in insecticide resistance. Differences between individual proportions were assessed using Marascuilo's procedure [65].

Binomial confidence intervals [66, 67] were calculated for species distribution and mortality to insecticides. Odds ratios were used to estimate the effect size of DNA marker assays in relation to resistance phenotype. Further, general linear models (GLM) with logit link function for a binomial dependent variable was used to model the effect of different mutations, sampling site, species and interaction between DNA resistance markers on survivorship.

Differences in mortality trend was determined by first grouping villages into three regions, eastern, central and western villages according to ecological zones identified by Caputo et al. [44]. Western villages consisted of A: Bessi, B: Ndemban Tenda, C: Chogen Wellingara and D: Yallal Ba, central villages were E: Sinchu Njengudi, F: Dongoro Ba, G: Sare Seedy and H: Ngedden, and eastern villages were J: Njaiyel, K: Madina Samako, L: Sare Wuro and M: Gunjur Koto.

Ethical clearance

This study was approved by Medical Research Council Unit (MRC) scientific coordinating committee and ethical clearance obtained from The Gambia Government/MRC Joint Ethics committee. Informed oral consent was obtained during village sensitization meetings.

Results

In the 2013 collection season, 1005 mosquitoes were tested using the WHO tube bioassay protocol [58]; 508 against 4 % DDT, 497 against 0.05 % deltamethrin. *An. gambiae s.l.* was sampled from all but two villages, Madina Samako and Chogen Wellingara (Fig. 2). Three members of the *An. gambiae* complex were identified: *An. gambiae s.s.*, *An. arabiensis*, and *An coluzzii* together with some *An. gambiae* s.s. x *An. coluzzii* hybrids (Fig. 1 and Additional file 1: Tables S2, S3, S4). Of the paired study villages, vector composition could only be compared in four pairs because the two remaining pairs lacked mosquitoes in one or both of the constituent villages. In three of the four village pairs, species composition varied between high and low transmission village pairs (Fig. 1).

Phenotypic resistance to DDT and deltamethrin in a WHO bioassay

There were significant interspecies differences in the 24 h post-exposure mortality to DDT and deltamethrin. For DDT, resistance was most pronounced in *An. gambiae s.s.*, with only 37 % mortality (95 % CI 29–46 %), compared to the other four species (Pearson Chi squared test, $\chi^2 = 194$, df = 3, $p < 0.001$) (Fig. 2). Further analysis showed significant differences in mortality except between *An. arabiensis* and *An. coluzzii* (Additional file 1: Tables S2, S3, S4). There were also significant differences in mortality between species following deltamethrin exposure (Pearson's Chi squared test, $\chi^2 = 44.94$, df = 3, $p < 0.001$). A significant difference in mortality was only observed when species were compared to *An. gambiae s.s.*, with the exception of *An. coluzzii* × *An. gambiae s.s* hybrids (Additional file 1: Tables S2, S3, S4).

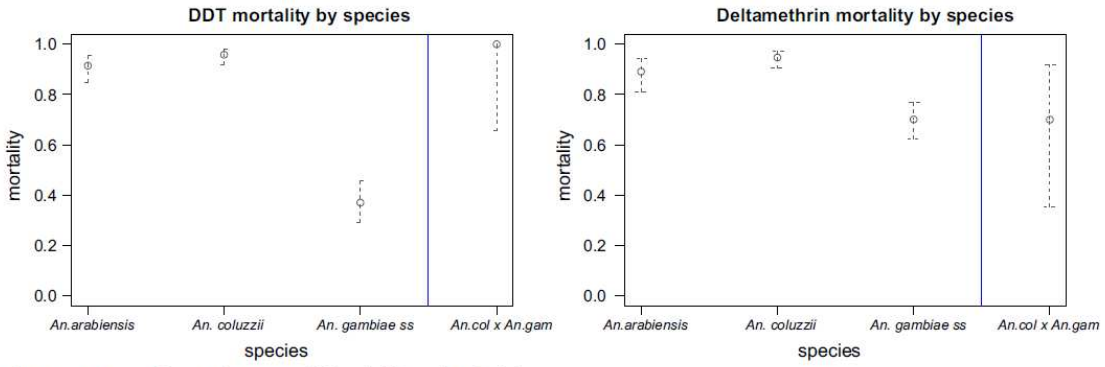

Fig. 2 Species-specific mortality to DDT (*left*) and deltamethrin (*right*)

There was a significant correlation between DDT and deltamethrin mortality (Kendall's correlation weighted by village, $\tau = 0.61$, $p = 0.02$), indicating that, *An. gambiae s.s.* populations were likely to be resistant to both insecticides.

There was variability in inter species mortality within and between villages for deltamethrin ($\chi^2 = 9.14$, $p = 0.03$) and DDT ($\chi^2 = 7.78$, $p = 0.05$). *An. gambiae s.s.* from the east were more resistant than those from the western part of the country (Table 1). DDT mortality tended to decrease from west to east, starting from Sinchu Njengudi (E). For deltamethrin, there was a similar trend though reduced mortality was mainly in Madina Samako (K), Sare Wuro (L) and Gunjur Koto (M) (Fig. 3).

Resistance association of DNA markers

The frequency of resistance alleles for various markers varied among species, with the *Vgsc-1014F* mutation being most common in *An. gambiae s.s.* (Table 2), and in this species there was a highly significant association with resistance to both DDT and deltamethrin. It was not possible to conduct these tests on the other species due to the low frequency of the *Vgsc-1014F* resistance mutation and high mortality.

In *An. gambiae s.s.*, survival of the *Gste2-114T* carriers was also significantly increased for DDT and (unexpectedly) for deltamethrin (Table 3). For *An. coluzzii*, there was no significant association between *Gste2-114T* and DDT resistance although a significant negative effect was observed for deltamethrin (Table 3).

Species, village and *Vgsc-1014F* explained significant variation in mortalities to both insecticides, though *Gste2-114T, Vgsc-1575Y* and interactions between markers were not significant (Table 4). Because of the absence of a sufficient number of survivors carrying resistance mutations in other species, other than *An. gambiae s.s.*, interaction between species and markers was not included in the model. A backward stepwise logistic regression therefore excluded *Gste2-114T* and *Vgsc-1575Y* in the final model (Additional file 1: Tables S3, S4). All the molecular markers screened in this study played a role in insecticide resistance but their effect was masked by the presence of the *Vgsc-1014F* mutation in captured *An. gambiae* s.s. which was a strong predictor of insecticide resistance.

Table 1 Differences in *Anopheles gambiae s.l.* mortality between east and western populations

Insecticide	Species	Region	Mortality (%)	χ^2	Df	P
DDT	*An. gambiae s.s.*	East	6	82.42	1	<0.001
		West	97			
	An. arabiensis	East	92	0.11	1	0.74
		West	97			
	An. coluzzii	East	67	4.75	1	0.03
		West	94			
Deltamethrin	*An. gambiae s.s.*	East	41	32.56	1	<0.001
		West	86			
	An. arabiensis	East	97	2.55	1	0.11
		West	83			
	An. coluzzii	East	89	0.04	1	0.85
		West	97			

Insecticide resistance and malaria transmission

For *An. gambiae s.s.*, mortality to DDT and deltamethrin was compared between high and low malaria prevalence villages. Data from the only village pair where there was no apparent difference in malaria infection rates ($p = 0.08$; villages G and H, central river region) are excluded as *An. coluzzii* was the only species collected. The unpaired Wilcoxon sum rank test was used because some villages did not have mortality data. DDT mortality

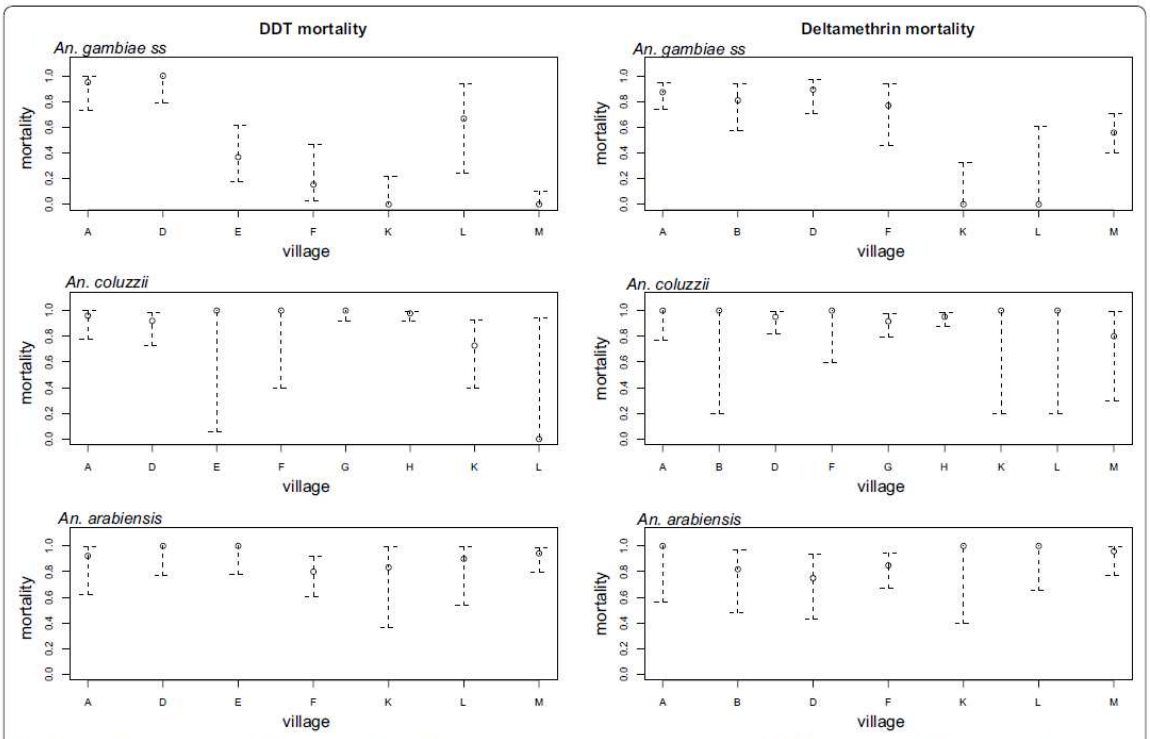

Fig. 3 Mortality by species and village (sampling site). Mortality against DDT (*left*), mortality against deltamethrin (*right*). Villages are labelled as in Fig. 1

Table 2 Allele frequency, in percentage expressed as proportions (allele/total number of alleles), of insecticide resistance mutations of malaria vectors in The Gambia

Species	*Vgsc-1014F*	*Vgsc-1014S*	*Vgsc-1575Y*	*Gste2-114T*	*Ace1-119S*
Allele frequency (with 95 % confidence intervals) of molecular resistance markers by species					
An. gambiae s.s.	0.51 (0.45–0.55)	0.004 (8.56^{-5}–0.01)	0.13 (0.1–0.16)	0.097 (0.075–0.12)	0.003 (5.68^{-4}–0.01)
An. arabiensis	0.05 (0.02–0.06)	0.14 (0.1–0.17)	0.002 (1.13^{-4}–0.01)	0.017 (0.008–0.04)	0 (0–0.1)
An. coluzzii	0.0012 (6.16^{-5}–7.62^{-3})	0 (0–0.01)	0.0012 (6.16^{-5}–0.01)	0.67 (0.67–0.74)	0 (0–0.01)
An. coluzzii × *An. gambiae* hybrid	0.15 (0.063–0.31)	0 (0–0.11)	0.08 (0.02–0.22)	0 (0–0.11)	0 (0–0.11)

for *An. gambiae s.s.* was significantly lower in high prevalence than low prevalence villages (Wilcoxon W = 0, p = 0.03). There was no observed difference in *An. gambiae s.s.* mortality to deltamethrin between high and low prevalence villages (W = 3.5, p = 0.24) or for any of the other species for both insecticides.

Discussion

Phenotypic resistance to DDT and deltamethrin was found mainly in *An. gambiae s.s.* and was more common in eastern Gambia where malaria transmission is higher than in the western regions [13, 68, 69], suggesting a link between insecticide resistance and observed malaria prevalence. Previous studies exploring the association between insecticide resistance and malaria endemicity have produced contrasting results, with some reporting no effect [70–73] while others suggesting otherwise [32]. In neighbouring Senegal [74] and in South Africa [35], following successful malaria control, increasing insecticide resistance coincided with higher incidence of clinical malaria. Nevertheless, proving a causal relationship between insecticide resistance and malaria transmission is extremely difficult [75].

Table 3 Odds ratios of *An. gambiae s.s.* and *An. coluzzii* mutants surviving an insecticide exposure for each insecticide resistance marker

Species	Insecticide	Marker	Odds ratio	95 % confidence intervals		P
				Lower	Upper	
An. gambiae s.s.	DDT	Kdr	253.74	48.07	6302.05	<0.001
		Gste2	3.4	1.43	9.18	0.01
	Deltamethrin	Kdr	8.37	3.99	18.47	<0.001
		Gste2	3.4	1.175	10.29	0.02
An. coluzzii	DDT	Gste2	1.5	0.34	11.35	0.72
	Deltamethrin	Gste2	0.23	0.06	0.78	0.02

Table 4 The effects of village, species and resistance markers on mortality of mosquitoes to DDT and deltamethrin using GLM

Factor	Df	Deviance	Residual Df	Residual deviance	P
DDT					
Species	5	190.56	490	331.83	<0.001
Village	8	114.2	482	217.62	<0.001
Kdr	5	51.04	477	166.58	<0.001
1575y	2	1.05	475	165.53	0.59
Gste2	2	1.8	473	163.73	0.41
Kdr:1575Y	1	0.86	472	162.87	0.35
kdr:gste2	4	4.33	468	158.55	0.36
1575Y:gste2	2	1.13E−08	466	158.55	1
Deltamethrin					
Species	5	50.46	482	371.69	<0.001
Village	8	34.45	474	337.24	<0.001
Kdr	5	28.22	469	309.02	<0.001
1575y	2	3.13	467	305.9	0.21
Gste2	2	4.26	465	301.63	0.12
Kdr:1575Y	1	0.09	464	301.54	0.76
Kdr:gste2	3	4.07	461	297.47	0.25
1575Y:gste2	3	0.86	458	296.61	0.83

Similar to earlier studies [68, 76, 77], three malaria vectors, namely *An. gambiae s.s.*, *An. coluzzii* and *An. arabiensis*, were observed across the country and in different proportions, in addition to a few hybrids of *An. gambiae s.s.* and *An. coluzzii*. *Anopheles melas*, known to breed in brackish water and usually found in western Gambia [44, 45, 78], was not collected. This may have been due to the rearing methods employed in the insectary.

The extreme interspecific differences observed in insecticide resistance status and frequency of mutations among them suggests that the involvement of insecticide resistance in malaria heterogeneity would be conditional on the vector species composition. This may help explain the differences in insecticide susceptibility estimates reported by two previous studies in eastern Gambia. In one study done in 2010, [43], *An gambiae s.l.* susceptibility to DDT and pyrethroids was about 90 % while in 2011 in a village of the same region, susceptibility to the same insecticides was only 50 % [42]. Such differences may be explained by the composition of the mosquito population tested. Indeed, in 2010, 70 % of all anophelines were *An. arabiensis*, while in 2011 this species represented only 42 % of all mosquitoes tested. Therefore, the high proportion of *An. arabiensis* may have concealed resistance in *An. gambiae s.s.*

Mechanisms of resistance

In *An. gambiae s.s.*, there was a clear association between the *Vgsc-1014F* mutation and phenotypic resistance, indicating that in The Gambia this is a very effective predictor of DDT and pyrethroids resistance. The *Vgsc-1575Y* and *Gste2-114T* markers had modest effects in conferring phenotypic resistance. Though in *An. gambiae s.s* and *An. arabiensis* the *Vgsc-1014S* mutation did not seem to be linked to phenotypic resistance, its low frequency limited statistical power. As in Uganda [79], few samples had both serine and phenylalanine mutations though carriers were also resistant to DDT. Given the low frequency of co-occurrence, it is not possible to establish whether carriage of both mutations confer an advantage, though this may be the case, at least compared to serine alone [80].

Population subdivision

The different insecticide resistance profile between eastern and western Gambia raises important questions about the drivers and stability of this heterogeneity. The GNMCP has distributed LLINs across the country since 2003 and sprayed houses yearly with DDT since 2008 [14], though only intermittently in the urban west coast region because of the lower malaria transmission. IRS has been carried out in all study villages so that DDT selection pressure should have been uniform. Nevertheless, intense DDT use in a community trial investigating the additional benefits of IRS with DDT to LLIN may have increased insecticide resistance pressure [43, 81].

With no history of carbamate and/or organophosphate use for public health in The Gambia, it is interesting to note that the two mosquitoes that had a carbamate/organophosphate resistance allele, *Ace1-119S*, were sampled from a village that is approximately 70 km from Guinguineo district, Senegal, where resistance to bendiocarb has been reported [82, 83], (President's Malaria Initiative, Senegal Report, unpublished), possibly linked to intense IRS campaigns with bendiocarb between 2008 and 2013. Investigation on the genetic connectivity between Gambian and Senegalese *An. gambiae* populations is currently underway.

Host seeking/foraging and resting behaviour of mosquitoes have been shown to play a role in the development of insecticide resistance [84, 85]. In The Gambia, the lack of detailed information on the behaviour of the sympatric malaria vectors limits proper insights into the causes of resistance in the eastern populations. Endophagy of *An. gambiae s.s.* may increase their exposure to insecticides, favouring the development of resistance [86, 87]. Conversely, exophagy of *An. arabiensis* [77] could play a role in the low levels of resistance observed in this species. However, in Senegal, where no difference in biting and host seeking behaviour were found [88] until recently [89], resistance has been reported mainly in *An. gambiae s.s.* and to a lesser extent in *An. arabiensis* and *An. coluzzii* [24, 89].

Conclusion

Insecticide resistance, which varies by species, seems to be associated to malaria endemicity although other factors not studied here may also be involved. Indeed, in eastern Gambia both insecticide resistance and malaria transmission are higher than in the rest of the country. The vector population is also extremely heterogeneous, underpinning the need for national malaria control programmes to continually monitor, as extensively as possible, the status of insecticide resistance to guide malaria control practices.

Additional file

Additional file 1: Table S1. Comparison of malaria prevalence rates between villages. **Table S2, S3, S4.** Statistical analysis of species specific mortality to DDT and deltamethrin.

Authors' contributions

KOO designed and conducted the study, performed laboratory work, analysed the data and wrote the manuscript; DW designed the study, assisted in data analysis, reviewed results and draft manuscripts including final version; MJ, MD, AF and FC assisted in coordinating field collection, rearing of samples, molecular laboratory work and reviewed the manuscript; JM assisted in coordinating field activities and reviewed the manuscript; UD, MJD designed the study, assisted in data analysis, reviewed results and draft manuscripts and final version of the manuscript for publication. All authors read and approved the final manuscript.

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Competing interests

The authors have declared that they have no competing interests.

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References

1. Gaudart J, Poudiougou B, Dicko A, Ranque S, Toure O, Sagara I, et al. Space-time clustering of childhood malaria at the household level: a dynamic cohort in a Mali village. BMC Public Health. 2006;6:286.
2. Ernst KC, Adoka SO, Kowuor DO, Wilson ML, John CC. Malaria hotspot areas in a highland Kenya site are consistent in epidemic and non-epidemic years and are associated with ecological factors. Malar J. 2006;5:78.
3. Kreuels B, Kobbe R, Adjei S, Kreuzberg C, von Reden C, Bäter K, et al. Spatial variation of malaria incidence in young children from a geographically homogeneous area with high endemicity. J Infect Dis. 2008;197:85–93.
4. Bejon P, Williams TN, Liljander A, Noor AM, Wambua J, Ogada E, et al. Stable and unstable malaria hotspots in longitudinal cohort studies in Kenya. PLoS Med. 2010;7:e1000304.
5. Macdonald G. The analysis of malaria epidemics. Trop Dis Bull. 1953;50:871.
6. WHO. World Malaria Report 2013. Geneva: World Health Organization. 2013.
7. WHO. Malaria elimination: a field manual for low and moderate endemic countries. Geneva: World Health Organization. 2007.
8. Woolhouse ME, Dye C, Etard J-F, Smith T, Charlwood J, Garnett G, et al. Heterogeneities in the transmission of infectious agents: implications for the design of control programs. Proc Natl Acad Sci USA. 1997;94:338–42.
9. Bousema T, Drakeley C, Gesase S, Hashim R, Magesa S, Mosha F, et al. Identification of hot spots of malaria transmission for targeted malaria control. J Infect Dis. 2010;201:1764–74.
10. Thomas C, Lindsay S. Local-scale variation in malaria infection amongst rural Gambian children estimated by satellite remote sensing. Trans R Soc Trop Med Hyg. 2000;94:159–63.
11. Burkot TR, Graves PM, Paru R, Wirtz RA, Heywood PF. Human malaria transmission studies in the *Anopheles punctulatus* complex in Papua New Guinea: sporozoite rates, inoculation rates, and sporozoite densities. Am J Trop Med Hyg. 1988;39:135–44.
12. Browne E, Frimpong E, Sievertsen J, Hagen J, Hamelmann C, Dietz K, et al. Malariometric update for the rainforest and savanna of Ashanti region. Ghana. Ann Trop Med Parasitol. 2000;94:15–22.

13. Mwesigwa J, Okebe J, Affara M, Di Tanna GL, Nwakanma D, Janha O, et al. On-going malaria transmission in The Gambia despite high coverage of control interventions: a nationwide cross-sectional survey. Malar J. 2015;14:314.
14. GNMCP. Malaria Indicator Survey. Banjul: National Malaria Control Programme. 2011.
15. Stoddard ST, Morrison AC, Vazquez-Prokopec GM, Soldan VP, Kochel TJ, Kitron U, et al. The role of human movement in the transmission of vector-borne pathogens. PLoS Negl Trop Dis. 2009;3:e481.
16. Klinkenberg E, McCall P, Wilson MD, Akoto AO, Amerasinghe FP, Bates I, et al. Urban malaria and anaemia in children: a cross-sectional survey in two cities of Ghana. Trop Med Int Health. 2006;11:578–88.
17. Wesolowski A, Eagle N, Tatem AJ, Smith DL, Noor AM, Snow RW, et al. Quantifying the impact of human mobility on malaria. Science. 2012;338:267–70.
18. Smith T, Charlwood J, Takken W, Tanner M, Spiegelhalter D. Mapping the densities of malaria vectors within a single village. Acta Trop. 1995;59:1–18.
19. Gillies M. Anopheline mosquitoes: vector behaviour and bionomics. Malaria: principles and practice of malariology. 1988;1:453–85.
20. Midega JT, Smith DL, Olotu A, Mwangangi JM, Nzovu JG, Wambua J, et al. Wind direction and proximity to larval sites determines malaria risk in Kilifi District in Kenya. Nat Comm. 2012;3:674.
21. Klinkenberg E, McCall P, Wilson MD, Amerasinghe FP, Donnelly MJ. Impact of urban agriculture on malaria vectors in Accra, Ghana. Malar J. 2008;7:151.
22. Ijumba JN, Mosha FW, Lindsay SW. Malaria transmission risk variations derived from different agricultural practices in an irrigated area of northern Tanzania. Med Vet Entomol. 2002;16:28–38.
23. Lindsay SW, Jawara M, Paine K, Pinder M, Walraven GEL, Emerson PM. Changes in house design reduce exposure to malaria mosquitoes. Trop Med Int Health. 2003;8:512–7.
24. Santolamazza F, Calzetta M, Etang J, Barrese E, Dia I, Caccone A, et al. Distribution of knock-down resistance mutations in Anopheles gambiae molecular forms in west and west-central Africa. Malar J. 2008;7:e74.
25. Edi CV, Koudou BG, Jones CM, Weetman D, Ranson H. Multiple-insecticide resistance in *Anopheles gambiae* mosquitoes, Southern Cote d'Ivoire. Emerg Infect Dis. 2012;18:1508.
26. Antonio-Nkondjio C, Fossog BT, Ndo C, Djantio BM, Togouet SZ, Awono-Ambene P, et al. *Anopheles gambiae* distribution and insecticide resistance in the cities of Douala and Yaounde(Cameroon): influence of urban agriculture and pollution. Malar J. 2011;10:154.
27. Dabire K, Baldet T, Diabate A, Dia I, Costantini C, Cohuet A, et al. *Anopheles funestus* (Diptera: culicidae) in a humid savannah area of western Burkina Faso: bionomics, insecticide resistance status, and role in malaria transmission. J Med Entomol. 2007;44:990–7.
28. Casimiro S, Coleman M, Hemingway J, Sharp B. Insecticide resistance in *Anopheles arabiensis* and *Anopheles gambiae* from Mozambique. J Med Entomol. 2006;43:276–82.
29. Corbel V, N'Guessan R, Brengues C, Chandre F, Djogbenou L, Martin T, et al. Multiple insecticide resistance mechanisms in *Anopheles gambiae* and *Culex quinquefasciatus* from Benin, West Africa. Acta Trop. 2007;101:207–16.
30. Coetzee M, van Wyk P, Booman M, Koekemoer LL, Hunt RH. Insecticide resistance in malaria vector mosquitoes in a gold mining town in Ghana and implications for malaria control. Bull Soc Path Exot. 2006;99:400–3.
31. Ochomo E, Bayoh NM, Kamau L, Atieli F, Vulule J, Ouma C, et al. Pyrethroid susceptibility of malaria vectors in four Districts of western Kenya. Parasit Vectors. 2014;7:310–8.
32. Zhou G, Afrane YA, Vardo-Zalik AM, Atieli H, Zhong D, Wamae P, et al. Changing patterns of malaria epidemiology between 2002 and 2010 in Western Kenya: the fall and rise of malaria. PLoS ONE. 2011;6:e20318.
33. N'Guessan R, Corbel V, Akogbeto M, Rowland M. Reduced efficacy of insecticide-treated nets and indoor residual spraying for malaria control in pyrethroid resistance area, Benin. Emerg Infect Dis. 2007;13:199–206.
34. Molineaux L, Shidrawi G, Clarke J, Boulzaguet J, Ashkar T. Assessment of insecticidal impact on the malaria mosquito's vectorial capacity, from data on the man-biting rate and age-composition. Bull World Health Organ. 1979;57:265.
35. Hargreaves K, Koekemoer L, Brooke B, Hunt R, Mthembu J, Coetzee M. *Anopheles funestus* resistant to pyrethroid insecticides in South Africa. Med Vet Entomol. 2000;14:181–9.
36. Garrett-Jones C, Grab B. The assessment of insecticidal impact on the malaria mosquito's vectorial capacity, from data on the proportion of parous females. Bull World Health Organ. 1964;31:71.
37. Busvine J, Pal R. The impact of insecticide-resistance on control of vectors and vector-borne diseases. Bull World Health Organ. 1969;40:731.
38. Busvine JR. Current problems in the control of mosquitoes. Nature. 1978;273:604–7.
39. SAMCP. Kwazulu/Natal Malaria Crisis Progress Report. South Africa: KwaZulu/Natal Department of Health Statistics. 2001.
40. Ceesay SJ, Casals-Pascual C, Nwakanma DC, Walther M, Gomez-Escobar N, Fulford AJ, et al. Continued decline of malaria in The Gambia with implications for elimination. PLoS ONE. 2010;5:e12242.
41. MoH. National Health Policy 2012–2020. In: Welfare MoHaS, editor. Banjul: Ministry of Health and Social Welfare; 2012. p. 22.
42. Tangena J, Adiamoh M, D'Alessandro U, Jarju L, Jawara M, Jeffries D, et al. Alternative treatments for indoor residual spraying for malaria control in a village with pyrethroid-and DDT-resistant vectors in the Gambia. PLoS ONE. 2013;8:e74351.
43. Pinder M, Jawara M, Jarju LB, Salami K, Jeffries D, Adiamoh M, et al. Efficacy of indoor residual spraying with dichlorodiphenyltrichloroethane against malaria in Gambian communities with high usage of long-lasting insecticidal mosquito nets: a cluster-randomised controlled trial. Lancet. 2014;385:1436–46.
44. Caputo B, Nwakanma D, Jawara M, Adiamoh M, Dia I, Konate L, et al. *Anopheles gambiae* complex along The Gambia river, with particular reference to the molecular forms of *An. gambiae s.s.* Malar J. 2008;7:182.
45. Majambere S, Fillinger U, Sayer DR, Green C, Lindsay SW. Spatial distribution of mosquito larvae and the potential for targeted larval control in The Gambia. Am J Trop Med Hyg. 2008;79:19–27.
46. Bryan J, Petrarca V, Di Deco M, Coluzzi M. Adult behaviour of members of the *Anopheles gambiae* complex in the Gambia with special reference to *An. melas* and its chromosomal variants. Parassitologia. 1986;29:221–49.
47. Reimer L, Tripet F, Slotman M, Spielman A, Fondjo E, Lanzaro G. An unusual distribution of the kdr gene among populations of *Anopheles gambiae* on the island of Bioko, Equatorial Guinea. Insect Mol Biol. 2005;14:683–8.
48. Dabiré KR, Diabaté A, Djogbenou L, Ouari A, N'Guessan R, Ouédraogo J-B, et al. Dynamics of multiple insecticide resistance in the malaria vector *Anopheles gambiae* in a rice growing area in South-Western Burkina Faso. Malar J. 2008;7:188.
49. Djègbè I, Boussari O, Sidick A, Martin T, Ranson H, Chandre F, et al. Dynamics of insecticide resistance in malaria vectors in Benin: first evidence of the presence of L1014S kdr mutation in *Anopheles gambiae* from West Africa. Malar J. 2011;10:261.
50. Costantini C, Ayala D, Guelbeogo WM, Pombi M, Some CY, Bassole IH, et al. Living at the edge: biogeographic patterns of habitat segregation conform to speciation by niche expansion in *Anopheles gambiae*. BMC Ecol. 2009;9:16.
51. Gimonneau G, Pombi M, Choisy M, Morand S, Dabiré RK, Simard F. Larval habitat segregation between the molecular forms of the mosquito *Anopheles gambiae* in a rice field area of Burkina Faso, West Africa. Med Vet Entomol. 2012;26:9–17.
52. Taylor C, Touré YT, Carnahan J, Norris DE, Dolo G, Traoré SF, et al. Gene flow among populations of the malaria vector, *Anopheles gambiae*, in Mali, West Africa. Genetics. 2001;157:743–50.
53. Clarkson CS, Weetman D, Essandoh J, Yawson AE, Maslen G, Manske M, et al. Adaptive introgression between *Anopheles* sibling species eliminates a major genomic island but not reproductive isolation. Nat Comm. 2014;5:4248.
54. Weetman D, Wilding CS, Steen K, Pinto J, Donnelly MJ. Gene flow–dependent genomic divergence between *Anopheles gambiae* M and S forms. Mol Biol Evol. 2012;29:279–91.
55. Chandre F, Manguin S, Brengues C, Dossou YJ, Darriet F, Diabate A, et al. Current distribution of a pyrethroid resistance gene (kdr) in *Anopheles gambiae* complex from west Africa and further evidence for reproductive isolation of the Mopti form. Parassitologia. 1999;41:319–22.

56. Weill M, Chandre F, Brengues C, Manguin S, Akogbeto M, Pasteur N, et al. The kdr mutation occurs in the Mopti form of *Anopheles gambiae s.s.* through introgression. Insect Mol Biol. 2000;9:451–5.
57. Majambere S, Lindsay SW, Green C, Kandeh B, Fillinger U. Microbial larvicides for malaria control in The Gambia. Malar J. 2007;6:76.
58. WHO. Test procedures for insecticide resistance monitoring in malaria vector mosquitoes. Geneva: World Health Organization. 2013.
59. Scott JA, Brogdon WG, Collins FH. Identification of single specimens of the *Anopheles gambiae* complex by the Polymerase Chain Reaction. Am J Trop Med Hyg. 1993;49:520–9.
60. Santolamazza F, Mancini E, Simard F, Qi Y, Tu Z, Della Torre A. Insertion polymorphisms of SINE200 retrotransposons within speciation islands of *Anopheles gambiae* molecular forms. Malar J. 2008;7:163.
61. Bass C, Williamson MS, Wilding CS, Donnelly MJ, Field LM. Identification of the main malaria vectors in the *Anopheles gambiae* species complex using a TaqMan real-time PCR assay. Malar J. 2007;6:155.
62. Bass C, Nikou D, Vontas J, Williamson MS, Field LM. Development of high-throughput real-time PCR assays for the identification of insensitive acetylcholinesterase (ace-1R) in *Anopheles gambiae*. Pest Biochem Physiol. 2010;96:80–5.
63. Jones CM, Liyanapathirana M, Agossa FR, Weetman D, Ranson H, Donnelly MJ, et al. Footprints of positive selection associated with a mutation (N1575Y) in the voltage-gated sodium channel of *Anopheles gambiae*. Proc Natl Acad Sci USA. 2012;109:6614–9.
64. Mitchell SN, Rigden DJ, Dowd AJ, Lu F, Wilding CS, Weetman D, et al. Metabolic and Target-Site Mechanisms Combine to Confer Strong DDT Resistance in *Anopheles gambiae*. PLoS ONE. 2014;9:e92662.
65. Marascuilo LA. Large-sample multiple comparisons. Psychol Bull. 1966;65:280.
66. Newcombe RG. Two-sided confidence intervals for the single proportion: comparison of seven methods. Stat Med. 1998;17:857–72.
67. Wilson EB. Probable inference, the law of succession, and statistical inference. J Am Stat Assoc. 1927;22:209–12.
68. Thomson M, d'Alessandro U, Bennett S, Connor S, Langerock P, Jawara M, et al. Malaria prevalence is inversely related to vector density in The Gambia, West Africa. Trans R Soc Trop Med Hyg. 1994;88:638–43.
69. Okebe J, Affara M, Correa S, Muhammad AK, Nwakanma D, Drakeley C, et al. School-Based Countrywide Seroprevalence Survey Reveals Spatial Heterogeneity in Malaria Transmission in the Gambia. PLoS ONE. 2014;9:e110926.
70. Henry M-C, Assi S-B, Rogier C, Dossou-Yovo J, Chandre F, Guillet P, et al. Protective efficacy of lambda-cyhalothrin treated nets in *Anopheles gambiae* pyrethroid resistance areas of Cote d'Ivoire. Am J Trop Med Hyg. 2005;73:859–64.
71. Wondji CS, Coleman M, Kleinschmidt I, Mzilahowa T, Irving H, Ndula M, et al. Impact of pyrethroid resistance on operational malaria control in Malawi. Proc Natl Acad Sci USA. 2012;109:19063–70.
72. Tokponnon FT, Ogouyémi AH, Sissinto Y, Sovi A, Gnanguenon V, Cornélie S, et al. Impact of long-lasting, insecticidal nets on anaemia and prevalence of *Plasmodium falciparum* among children under five years in areas with highly resistant malaria vectors. Malar J. 2014;13:76.
73. Lindblade KA, Mwandama D, Mzilahowa T, Steinhardt L, Gimnig J, Shah M, et al. A cohort study of the effectiveness of insecticide-treated bed nets to prevent malaria in an area of moderate pyrethroid resistance, Malawi. Malar J. 2015;14:31.
74. Trape J-F, Tall A, Diagne N, Ndiath O, Ly AB, Faye J, et al. Malaria morbidity and pyrethroid resistance after the introduction of insecticide-treated bednets and artemisinin-based combination therapies: a longitudinal study. Lancet Infect Dis. 2011;11:925–32.
75. Kleinschmidt I, Mnzava AP, Kafy HT, Mbogo C, Bashir AI, Bigoga J, et al. Design of a study to determine the impact of insecticide resistance on malaria vector control: a multi-country investigation. Malar J. 2015;14:282.
76. Jawara M, Pinder M, Drakeley CJ, Nwakanma DC, Jallow E, Bogh C, et al. Dry season ecology of *Anopheles gambiae* complex mosquitoes in The Gambia. Malar J. 2008;7:9.
77. Lindsay S, Alonso P, Schellenberg JA, Hemingway J, Adiamah J, Shenton F, et al. A malaria control trial using insecticide-treated bed nets and targeted chemoprophylaxis in a rural area of The Gambia, west Africa 7. Impact of permethrin-impregnated bed nets on malaria vectors. Trans R Soc Trop Med Hyg. 1993;87(Suppl 2):45–51.
78. Bryan J, Di Deco M, Petrarca V, Coluzzi M. Inversion polymorphism and incipient speciation in *Anopheles gambiae s.s.* in the Gambia, West Africa. Genetica. 1982;59:167–76.
79. Mawejje HD, Wilding CS, Rippon EJ, Hughes A, Weetman D, Donnelly MJ. Insecticide resistance monitoring of field-collected Anopheles gambiae s.l. populations from Jinja, eastern Uganda, identifies high levels of pyrethroid resistance. Med Vet Entomol. 2013;27:276–83.
80. Reimer L, Fondjo E, Patchoke S, Diallo B, Lee Y, Ng A, et al. Relationship between *kdr* mutation and resistance to pyrethroid and DDT insecticides in natural populations of *Anopheles gambiae*. J Med Entomol. 2008;45:260–6.
81. Pinder M, Jawara M, Jarju LB, Kandeh B, Jeffries D, Lluberas MF, et al. To assess whether indoor residual spraying can provide additional protection against clinical malaria over current best practice of long-lasting insecticidal mosquito nets in The Gambia: study protocol for a two-armed cluster-randomised trial. Trials. 2011;12:147.
82. SNMCP. Profil entomologique du paludisme au Senegal: Ministere de la Sante et de la prevention Medicale Profil. 2011.
83. Niang E. A. Variabilité génétique des populations d'*Anopheles gambiae s.l.* au Sénégal: implications dans le contrôle du paludisme. Cheikh Anta Diop de Dakar. 2014.
84. Padonou G, Sezonlin M, Gbedjissi G, Ayi I, Azondekon R, Djenontin A, et al. Biology of *Anopheles gambiae* and insecticide resistance: entomological study for a large scale of indoor residual spraying in south east Benin. J Parasitol Vector Biol. 2011;3:59–68.
85. Kloke RG, Nhamahanga E, Hunt RH, Coetzee M. Vectorial status and insecticide resistance of *Anopheles funestus* from a sugar estate in southern Mozambique. Parasit Vectors. 2011;4:16.
86. Stump AD, Atieli FK, Vulule JM, Besansky NJ. Dynamics of the pyrethroid knockdown resistance allele in western Kenyan populations of *Anopheles gambiae* in response to insecticide-treated bed net trials. Am J Trop Med Hyg. 2004;70:591–6.
87. de Zulueta J. Insecticide resistance in *Anopheles sacharovi*. Bull World Health Organ. 1959;20:797.
88. Diatta M, Spiegel A, Lochouarn L, Fontenille D. Similar feeding preferences of *Anopheles gambiae* and *A. arabiensis* in Senegal. Trans R Soc Trop Med Hyg. 1998;92:270–2.
89. Ndiath MO, Mazenot C, Sokhna C, Trape JF. How the malaria vector *Anopheles gambiae* adapts to the use of insecticide-treated nets by African populations. PLoS ONE. 2014;9:e97700.

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Appendix 2 Prevalence of asymptomatic *Plasmodium falciparum* as determined by PCR by site and village in 2012 cross-sectional survey

region	Village	village code	no. positive	N	*P.falciparum* prevalence	Prop.test (χ2)	p value
1	Bessi	A	5	308	1.6	9	0.003
	Ndemban Tenda	B	11	141	7.8		
2	Chogen Wellingara	C	21	205	10.2	14.9	0
	Yallal Ba	D	6	302	1.9		
3	Dongoro Ba	F	45	300	15	6	0.015
	Sinchu Njengudi	E	5	100	5		
4	Ngedden	H	3	180	1.7	3.1	0.08
	Sare Seedy	G	17	321	5.3		
5	Njaiyel	J	65	307	21.2	53.4	0
	Madina Samako	K	169	345	48.8		
6	Sare Wuro	L	69	247	27.9	9.6	0.002
	Gunjur Koto	M	86	203	42.4		

Data from Mwesigwa *et al* (Mwesigwa et al., 2015). Prop.test is paired test of proportions between villages in the same region

Appendix 3 Species distribution and composition as sampled by larvae and house searches in 2013.

Village	Village code	*An. arabiensis*	*An. coluzzii*	*M/S* Hybrids	*An. gambiae s.s.*	total
Bessi	A	20	42	10	67	139
Ndemban Tenda	B	12	2	2	21	37
Yallal Ba	D	29	66	3	47	145
Sinchu Njengudi	E	18	1	0	19	38
Dongoro Ba	F	63	13	4	26	106
Sare Seedy	G	0	105	0	0	105
Ngedden	H	0	218	0	0	218
Madina Samako	K	10	13	0	29	52
Sare Wuro	L	20	3	0	10	33
Gunjur Koto	M	59	5	1	86	151

Appendix 4 Species specific mortalities to DDT and deltamethrin performed using Marascuilo procedure

Proportion pairs	Value (difference in proportions)	Critical range	
DDT			
P1-P2	0.044	0.084	Not significant
P1-P3	0.545	0.137	Significant
P1-P4	0.085	0.069	Significant
P2-P3	0.589	0.099	Significant
P2-P4	0.041	0.038	Significant
P3-P4	0.63	0.427	Significant
Deltamethrin			
P1-P2	0.05	0.097	Not significant
P1-P3	0.192	0.131	Significant
P1-P4	0.192	0.414	Not significant
P2-P3	0.242	0.109	Significant
P2-P4	0.243	0.408	Not significant
P3-P4	0.001	0.417	Not significant

P1-An. arabiensis, *P2- An. coluzzii*, *P3- An. gambiae s.s.*, *P4- An. coluzzii* × *An. gambiae s.s.* hybrid. The test statistic is the absolute difference between estimated mortality proportions. If value (pair wise difference in proportions) is higher than critical range, difference is considered significant.

Appendix 5 Differences in *An. gambiae s.l* mortality between east and western populations

Insecticide	Species	Region	Mortality (%)	χ^2	Df	P
DDT	*An. gambiae ss*	east	6	82.42	1	<0.001
		west	97			
	An. arabiensis	east	92	0.11	1	0.74
		west	97			
	An. coluzzii	east	67	4.75	1	0.03
		west	94			
Deltamethrin	*An. gambiae ss*	east	41	32.56	1	< 0.001
		west	86			
	An. arabiensis	east	97	2.55	1	0.11
		west	83			
	An. coluzzii	east	89	0.04	1	0.85
		west	97			

Appendix 6 Generalized Linear model testing the effects of village, species and kdr on mortality of mosquitoes to DDT.

Factors	**Factor levels**	**Estimate**	**Std. Error**	**z.value**	**p**
	(Intercept)	-3.9	0.86	-4.53	< 0.001
Village	Yallal Ba	-0.79	0.99	-0.79	0.428
	Sinchu Njengudi	0.3	1.02	0.3	0.765
	Dongoro Ba	0.95	0.95	1	0.319
	Sare Seedy	-17.12	1410.25	-0.01	0.99
	Ngedden	-1.26	0.98	-1.29	0.199
	Madina Samako	1.8	0.85	2.11	0.035
	Sare Wuro	-2.09	1.39	-1.51	0.132
	Gunjur Koto	0.88	1.03	0.86	0.392
Species	*An. coluzzii*	1.36	0.77	1.76	0.079
	An.col-An.gam hybrid	-16.45	3063.43	-0.01	0.996
	An. gambiae s.s.	0.87	0.85	1.03	0.302
Kdr	*Kdr: FF*	5.77	1.13	5.1	< 0.001
	Kdr: FS	4.47	1.39	3.21	< 0.01
	Kdr: LF	2.08	0.89	2.33	0.02
	Kdr: LS	1.63	0.86	1.88	0.06
	Kdr: SS	-15.99	7257.14	0	0.998

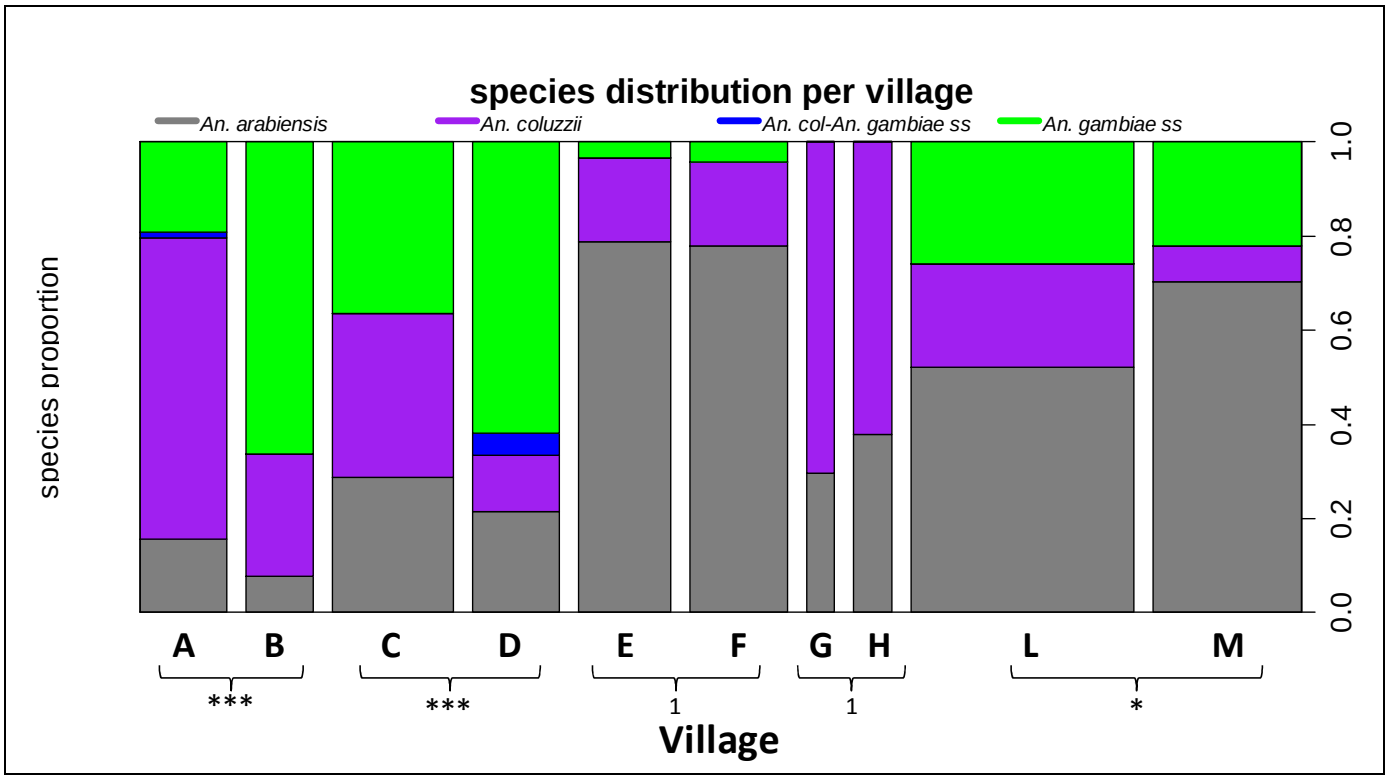
Kdr refers to Knockdown resistance gene. Letters denote amino acid subsititutions at the 1014 codon; *FF*- homozygous for phenylalanine, *FS* has both phenylalanine and serine, *LF* – heterozygous for phenylalanine, *LS* – heterozygous for serine and *SS* – homozygous for serine

Appendix 7 Generalized Linear model testing the effects of village, species and kdr on mortality of mosquitoes to deltamethrin

Factor	Factor levels	Estimate	Std. Error	z.value	p
	(Intercept)	-3.1	0.69	-4.46	< 0.001
Village	Bessi	1.36	0.66	2.05	0.041
	Yallal Ba	0.97	0.65	1.5	0.135
	Dongoro Ba	0.7	0.67	1.04	0.3
	Sare Seedy	1.59	0.95	1.68	0.093
	Ngedden	1.01	0.94	1.07	0.285
	Madina Samako	1.94	0.95	2.04	0.042
	Sare Wuro	0.47	0.97	0.48	0.631
	Gunjur Koto	-0.11	0.74	-0.15	0.884
Species	*An. coluzzii*	-0.89	0.77	-1.16	0.246
	*An. col - An. gam s.s.*hybrid	0.5	0.92	0.55	0.584
	An. gambiae s.s.	0.28	0.55	0.52	0.603
kdr	*Kdr: FF*	2.99	0.68	4.42	< 0.001
	Kdr: FS	-12.43	882.74	-0.01	0.989
	Kdr: LF	1.8	0.53	3.4	< 0.001
	Kdr: LS	0.06	0.86	0.07	0.947
	Kdr: SS	2.52	1.52	1.65	0.098

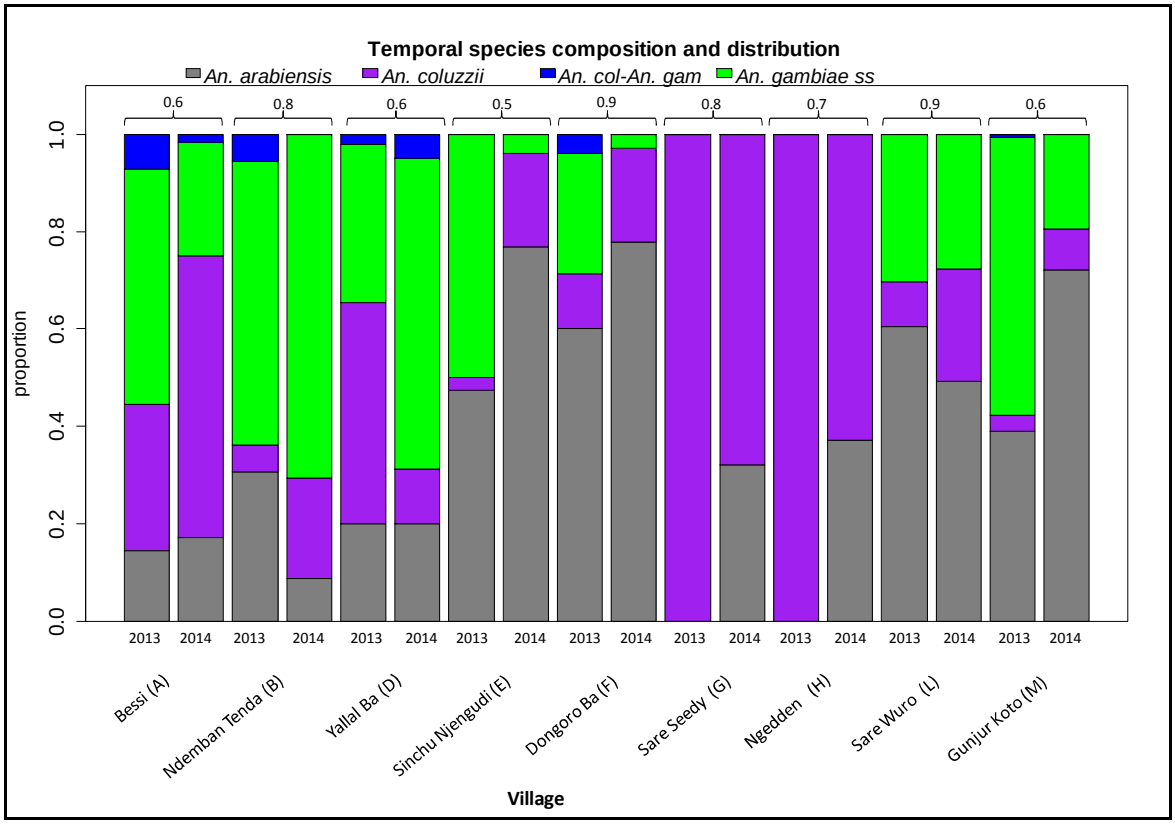
Kdr refers to Knockdown resistance gene. Letters denote amino acid subsititutions at the 1014 codon; *FF*- homozygous for phenylalanine, *FS* has both phenylalanine and serine, *LF* – heterozygous for phenylalanine, *LS* – heterozygous for serine and *SS* – homozygous for serine

Appendix 8 Species composition and proportion of malaria vectors across The Gambia in 2014



Species composition and proportion of malaria vectors across The Gambia in 2014. Asterisks denote significant differences in species diversity per year assessed using Chi-squared tests; *** p< 0.001, * p< 0.05 and 1 no significant difference. Letters denote villages; A- Bessi, B – Ndemban Tenda, C – Chogen wellingara, D – Yallal Ba, E – Sinchu Njengudi, F – Dongoro Ba, G – Sare Seedy, H – Ngedden, L – Sare Wuro and M – Gunjur Koto.

Appendix 9 Species composition and proportion of malaria vectors sampled from larvae in the transmission seasons of 2013 and 2014 across The Gambia



Species composition and proportion of malaria vectors sampled from larvae in the transmission seasons of 2013 and 2014 across Numbers denote Yue and Clayton similarity index, θ, of species proportions between pairs of two communities. Zero (0) denote dissimilarity while 1 indicates similarity in species composition based on proportions.. An col-An *gambiae* ss refer to hybrid between *An. coluzzii* and *An. gambiae s.s*

Appendix 10 Species composition of blood-fed anophelines collected using CDC-LT in the transmission season of 2013 in The Gambia.

Village	Village code	*An. arabiensis*	*An. funestus*	*An. coluzzii*	*An. melas*	*M/S* Hybrids	*An. gambiae s.s.*	Total
Bessi	A	2	0	3	1	0	31	37
Ndemban Tenda	B	3	0	0	0	0	6	9
Chogen wellingara	C	4	0	1	2	0	18	25
Yallal Ba	D	3	0	1	0	2	12	18
Sinchu Njengudi	E	14	0	30	31	0	1	76
Dongoro Ba	F	13	0	6	11	0	2	32
Sare Seedy	G	9	82	26	0	0	2	119
Ngedden	H	2	244	10	0	0	0	256
Madina Samako	J	14	0	1	1	0	21	37
Sare Wuro	K	7	0	2	0	0	28	37
Gunjur Koto	L	10	0	1	0	1	26	38
	M	12	0	1	0	0	16	29

Appendix 11 Species composition of malaria vectors sampled from larvae in the transmission seasons of 2013 and 2014 across The Gambia.

Village	Village code	Year	*An. arabiensis*	*An. coluzzii*	*An. gambiae s.s.*	*M/S* Hybrids	Total
Bessi	A	2013	20	42	67	10	139
		2014	11	37	15	1	64
Ndemban Tenda	B	2013	11	2	21	2	36
		2014	5	12	41	0	58
Yallal Ba	D	2013	29	66	47	3	145
		2014	16	9	51	4	80
Sinchu Njengudi	E	2013	18	1	19	0	38
		2014	60	15	3	0	78
Dongoro Ba	F	2013	63	12	26	4	105
		2014	53	13	2	0	68
Sare Seedy	G	2013	0	105	0	0	105
		2014	8	17	0	0	25
Ngedden	H	2013	0	175	0	0	175
		2014	13	22	0	0	35
Sare Wuro	L	2013	20	3	10	0	33
		2014	91	43	51	0	185
Gunjur Koto	M	2013	59	5	86	1	151
		2014	93	11	25	0	129

Appendix 12 Generalized Linear Mixed Effect Model testing the effects of *Vgsc-1014* (F&S), *Vgsc-N1575Y, Gste2-114T*, species, region and year and village as a random effect on bioassay mortality to DDT. Estimates are log odds (logit) of surviving DDT exposure.

	Estimate	Std. Error	z value	p	
(Intercept)	-3.72	0.56	-6.69	<0.001	***
1014 FF	3.32	0.55	6.04	<0.001	***
1014 FS	3.94	0.8	4.93	<0.001	***
1014 LF	1.47	0.56	2.62	<0.01	**
1014 LS	1.52	0.57	2.67	<0.01	**
1014 SS	3.92	0.88	4.47	<0.001	***
Region2	-0.86	0.54	-1.57	0.116	
Region3	0.16	0.52	0.31	0.754	
Region4	-2.51	0.83	-3.02	<0.01	**
Region5	1.67	0.68	2.46	<0.05	*
Region6	0.77	0.51	1.51	0.132	
An. coluzzii	1.01	0.68	1.5	0.133	
An. gambiae s.s	1.47	0.53	2.79	<0.01	**
year2	-0.88	0.31	-2.88	<0.01	**
Gste2 - IT	0.35	0.51	0.69	0.489	
Gste2 - TT	0.99	0.57	1.74	0.081	.
1575Y -NY	1.16	0.63	1.85	0.065	.
1575Y YY	-0.05	0.88	-0.06	0.954	

Appendix 13 Generalized Linear Mixed Effect model testing the effect of *Vgsc*-1014F, region and year and village (random effect) on *An. gambiae s.s.* 24 hour post exposure mortality to deltamethrin

Fixed terms	Chisq	Df	p
Vgsc-1014F	8.65	2	<0.05
Region	1.23	3	0.745
Year	5.11	1	<0.05

Appendix 14 Malaria prevalence in 2014 as estimated by PCR

Village (Code)	Prevalence
Bessi (A)	1.9
Ndemban Tenda (B)	1.5
Chogen wellingara (C)	2.3
Yallala Ba (D)	1.5
Sinchu Njengudi (E)	1.6
Dongoro Ba (F)	2.3
Sare seedy (G)	2.6
Ngedden (H)	1.8
Njaiyel (J)	2.4
Madina Samako (K)	6.8
Sare Wuro (L)	8.3
Gujur koto (M)	8.4

Appendix 15 Temporal allele frequency (proportions with 95% confidence intervals) of resistance mutation markers in malaria vectors of The Gambia from 2013 - 2014

species	region	year	*Vgsc - 1014F*	*Vgsc - 1014S*	*Vgsc - 1575Y*	*Gste2-114T*	*Ace-1 119S*
An. gambiae ss	1	2013	0.08 (0.05-0.13)		0.01 (0-0.04)	0.01 (0-0.04)	
		2014	0.04 (0.02-0.1)			0.04 (0.01-0.11)	
	2	2013	0.02 (0-0.08)		0.16 (0.09-0.25)	0.2 (0.13-0.3)	
		2014	0.15 (0.11-0.22)		0.26 (0.16-0.39)	0.19 (0.1-0.32)	
	3	2013	0.61 (0.5-0.71)		0.25 (0.19-0.32)	0.13 (0.08-0.18)	
		2014	0.21 (0.06-0.51)		0.02 (0-0.07)	0.03 (0.01-0.08)	
	5	2013	1 (0.92-1)		0.03 (0.01-0.06)	0.02 (0-0.05)	
		2014				0.2 (0.04-0.56)	
	6	2013	0.95 (0.9-0.97)				0.01 (0-0.04)
		2014	0.86 (0.79-0.9)		0.19 (0.13-0.26)	0.13 (0.09-0.2)	0.07 (0.04-0.12)
An. coluzzii	1	2013				0.91 (0.82-0.96)	
		2014				0.9 (0.82-0.95)	
	2	2013				0.72 (0.63-0.79)	
		2014			0.01 (0-0.06)	0.88 (0.79-0.93)	
	3	2013				0.42 (0.24-0.63)	
		2014				0.72 (0.59-0.83)	
	4	2013	0 (0-0.01)		0 (0-0.01)	0.69 (0.65-0.73)	
		2014	0.02 (0-0.09)		0.01 (0-0.08)	0.45 (0.34-0.56)	
	5	2013				0.46 (0.27-0.66)	
		2014					
	6	2013				0.88 (0.6-0.98)	
		2014				0.92 (0.85-0.96)	
An. arabiensis	1	2013					
		2014	0.17 (0.07-0.33)	0.33 (0.19-0.51)			
	2	2013	0.05 (0.01-0.15)	0.22 (0.13-0.36)		0.14 (0.07-0.26)	
		2014	0.1 (0.05-0.18)	0.22 (0.14-0.31)	0.02 (0-0.08)		
	3	2013	0.05 (0.02-0.1)	0.18 (0.12-0.25)	0.01 (0-0.04)		
		2014	0.02 (0.01-0.05)	0.28 (0.23-0.34)		0.07 (0.04-0.11)	
	6	2013	0.04 (0.02-0.08)	0.1 (0.06-0.16)			
		2014	0.05 (0.03-0.08)	0.2 (0.16-0.24)	0.01 (0-0.03)		

An. agambiess X An. coluzzii	1	2014			1 (0.2-1)
	2	2014	0.38 (0.1-0.74)		0.12 (0.01-0.53)
				0.38 (0.1-0.74)	
	3	2013	0.62 (0.26-0.9)		
	6	2013	0.5 (0.09-0.91)		

Missing data are NA- no mosquito samples were collected.

Appendix 16 Blood meal sources of malaria vectors collected using CDC-LT in 12 villages across The Gambia in the transmission seasons of 2013

species	**human**	**cow**	**donkey**	**goat**	**dog**	**human/ goat**	**human/ cow**	**cow/ goat**	**cow/ dog**
An. *gambiae* s.s.	64	1	3	5	0	5	0	0	0
An. arabiensis	30	3	6	2	0	2	1	3	0
An. funestus	22	107	0	16	3	1	0	0	1
An. *coluzzii*	36	4	12	1	0	0	0	0	0
An. melas	11	3	4	0	0	1	0	0	0
An. gam - An col hybrid	1	0	0	0	0	0	0	0	0

Appendix 17 Distribution of blood meal sources among malaria vectors sampled collected using CDC-LT during the transmission season of 2013.

			Vertebrate host									
								Others				
species	Village	Village code	Human	Cow	Goat	Donkey	Dog	Mix human_goat	Mix cow_human	Mix cow_dog	Mix cow_goat	Total
An. gambiae s.s.	Bessi	A	21	0	0	0	0	0	0	0	0	21
	Ndemban Tenda	B	5	0	0	0	0	0	0	0	0	5
	Chogen Wellingara	C	2	1	0	2	0	0	0	0	0	5
	Yallal Ba	D	4	0	1	1	0	0	0	0	0	6
	Sinchu Njengudi	E	1	0	0	0	0	0	0	0	0	1
	Dongoro ba	F	1	0	0	0	0	0	0	0	0	1
	Njayel	J	8	0	0	0	0	2	0	0	0	10
	Madina Samako	K	11	0	1	0	0	3	0	0	0	15
	Sare Wuro	L	6	0	2	0	0	0	0	0	0	8
	Gunjur Koto	M	5	0	1	0	0	0	0	0	0	6
An. coluzzii	Bessi	A	2	0	0	0	0	0	0	0	0	2
	Chogen Wellingara	C	1	0	0	0	0	0	0	0	0	1
	Sinchu Njengudi	E	18	0	0	2	0	0	0	0	0	20

	Dongoro ba	F	3	0	0	1	0	0	0	0	0	4
	Sare Seedy	G	8	2	1	7	0	0	0	0	0	18
	Ngedden	H	4	2	0	2	0	0	0	0	0	8
An. arabiensis	Chogen Wellingara	C	2	0	0	0	0	0	0	0	0	2
	Yallal Ba	D	0	0	0	2	0	0	0	0	0	2
	Sinchu Njengudi	E	7	1	0	0	0	0	0	0	0	8
	Dongoro ba	F	5	0	1	2	0	0	0	0	0	8
	Sare Seedy	G	4	1	0	2	0	0	0	0	0	7
	Njayel	J	5	0	0	0	0	0	0	0	0	5
	Madina Samako	K	1	0	0	0	0	1	0	0	0	2
	Sare Wuro	L	2	0	1	0	0	0	0	0	0	3
	Gunjur Koto	M	4	1	0	0	0	1	0	0	0	6
An. funestus	Sare Seedy	G	4	24	4	0	0	0	0	0	0	32
	Ngedden	H	18	83	12	0	3	1	1	1	3	122

Others: Refer to samples that had two sources of blood meals

Appendix 18 Anthropophilic rate (proportion of human blood relative to other hosts) between high and low malaria prevalence villages of blood fed malaria vectors sampled from 12 villages across The Gambia

Malaria prevalence	Species	Anthropophilic rate, %						Wilcoxon signed rank test	p
High		B	C	F	G	K	M		
	An. *gambiae* s.s.	1	0.4	1	NA	0.73	0.83	paired¥	0.79
	An. *coluzzii*		1	0.75	0.44	NA	NA	Unpaired †	0.75
	An. *arabiensis*		1	0.63	0.57	0.5	0.67	Unpaired #	0.73
low		A	D	E	H	J	L		
	An. *gambiae* s.s.	1	0.67	1	NA	0.8	0.75	¥	
	An. *coluzzii*	1	NA	0.9	0.5	NA	NA	†	
	An. *arabiensis*		0	0.87		1	0.67	#	

Letters denote villages as Figure 1. The ¥ denote paired test between *An. gambiae s.s.* anthropophilic rate in low and high prevalence villages, † unpaired for *An. coluzzii* and # unpaired for *An. arabiensis.*

Appendix 19 Association between blood meal host choice and molecular markers of resistance in *An. gambiae s.s* from eastern Gambia

		***Vgsc-L1014F* genotype**				
	N	Leu/Leu	Phe/Leu	Phe/Phe	Test statistic	P
Human positive	40	9	31	0	Exact	0.712
Bovine positive	13	4	9	0		
		Vgsc-N1575Y				
		Asn/Asn	Asn/Tyr	Tyr/Tyr		
Human positive	42	29	12	1	Exact	1
Bovine positive	13	9	4	0		
		Gste2-114T				
		Ile/Ile	Ile/Thr	Thr/Thr		
Human positive	42	37	4	1	Exact	0.376
Bovine positive	13	10	3	0		

Fisher's Exact test was done based on genotype, heterozygotes were grouped with homozygous mutants and compared against wild type allele. Leu (Leucine), Asn (Asparagine) and Ile (Isoleucine) are the wild type alleles while Phe (phenylalanine), Tyr (Tyrosine) and Thr (Threonine) are the resistance mutations.

Appendix 20 Species distribution of samples collected by HLC between Septermber-October 2013

Village	Village code	*An. arabiensis*	*An. coluzzii*	*An. gambiae* s.s.
Ndemban Tenda	B	1	0	11
Sinchu Njengudi	E	7	5	2
Ngedden	H	2	11	1
Sare Wuro	L	20	2	90
Gunjur Koto	M	28	1	70

Appendix 21 Association between genotype and phenotypic resistance of *An. gambiae* s.l. of mixed ages to DDT and Bendiocarb

species	Insecticide	marker	Odds Ratio	95% confidence intervals		P
				Lower	Upper	
An gambiae ss	DDT	*Vgsc*-1014F	18	2	557	0.01
		GSTe2	1	0.3	5.7	0.7
		N1575Y	0.7	0.2	2	0.5
	Bendiocarb	*Ace-1 119S*	41	4	1393	0.002
An arabiensis	DDT	*Vgsc*-1014F	4.7	0.1	76	0.3

Appendix 22 Characteristics of *Anopheles gambiae s l* from eastern villages; Sare Wuro and Gunjur Koto that tested positive for malaria parasites

species	**Infection status**	**phenotype**	**Parity**	
An. gambiae s.s	*P. Falciparum* positive	alive	Parous	5
			Gravid	1
		dead	Parous	2
			Gravid	3
An arabiensis	*Povm*	dead	Parous	1

Appendix 23 Summary of MICRO-CHECKER output assessing genotyping errors due null alleles, large allele dropouts and starter peaks

Population	loci	Stuttering error	Large allele dropout	Null alleles	Homozygous excess	Comment	Significant probability
CRR	AG3H242	no	no	no			
	AG3H128	no	no	no			NO
	AG3H93	yes	no	may be present ;excess homozygous	yes	alleles of one unit repeat units, check scoring	YES
	AGXH678	no	no	may be present ;excess homozygous	yes		
	AG3H119	yes	no	may be present ;excess homozygous	yes		YES
	AG3H555	no	no	no			NO
	AG3H249	no	no	no			NO
	XND5D1	no	no	may be present ;excess homozygous	yes		

	AG3H59	no	no	may be present ;excess homozygous	yes		
	AG3H577	no	no	may be present ;excess homozygous	yes		
	AG3H758		no		yes	alleles of one unit repeat units, check scoring	
	XND5C1	no	no	no			
	XND6U2	no	no	no			can't be computed:>50% are one size class
GamEast	AG3H242	no	no	may be present ;excess homozygous			YES
	AG3H128	no	no	may be present ;excess homozygous	yes		YES
	AG3H93	yes	no	may be present ;excess homozygous	yes	alleles of one unit repeat units, check scoring	YES
	AGXH678	no	no	may be present	yes		YES

			;excess homozygous			
AG3H119	no	no	may be present ;excess homozygous	yes		YES
AG3H555	no	no	may be present ;excess homozygous	yes		YES
AG3H249	no	no	no	no		NO
XND5D1	no	no	no	no		NO
AG3H59	no	no	may be present ;excess homozygous	yes		YES
AG3H577	no	no	may be present ;excess homozygous	yes		YES
AG3H758	yes	no	may be present ;excess homozygous	yes	alleles of one unit repeat units, check scoring	YES
XND5C1	no	no	may be present ;excess homozygous	yes		YES

	XND6U2	no	no	may be present ;excess homozygous	yes		YES
GamWest	AG3H242	no	no	may be present ;excess homozygous	yes		YES
	AG3H128	no	no	may be present ;excess homozygous	yes		YES
	AG3H93	yes	no	yes	yes	alleles of one unit repeat units, check scoring	YES
	AGXH678	no	no	may be present ;excess homozygous	yes		YES
	AG3H119	no	no	may be present ;excess homozygous	yes		YES
	AG3H555	yes	no	yes	yes	alleles of one unit repeat units, check scoring	YES
	AG3H249	no	no	no	no		NO

	XND5D1	no	no	no	no	can't be computed:>50% are one size class
	AG3H59	no	no	may be present ;excess homozygous	yes	YES
	AG3H577	no	no	may be present ;excess homozygous	yes	YES
	AG3H758	no	no	may be present ;excess homozygous	yes	YES
	XND5C1	no	no	may be present ;excess homozygous	yes	YES
	XND6U2	no	no	may be present ;excess homozygous	yes	YES
SenBadi	AG3H242	no	no	no	no	can't be computed:>50% are one size class
	AG3H128	no	no	no	no	NO
	AG3H93	no	no	may be present ;excess	yes	NO

			homozygous		
AGXH678	no	no	no	no	NO
AG3H119	no	no	may be present ;excess homozygous	yes	NO
AG3H555	no	no	no	no	NO
AG3H249	no	no	no	no	NO
XND5D1	no	no	no	no	NO
AG3H59	no	no	no	no	NO
AG3H577	no	no	no	no	can't be computed:>50% are one size class
AG3H758	no	no	no	no	can't be computed:>50% are one size class
XND5C1	no	no	may be present ;excess homozygous	yes	YES
XND6U2	no	no	may be present ;excess homozygous	yes	YES

SenBerk	AG3H242	no	no	no	no	can't be computed:>50% are one size class
	AG3H128	no	no	no	no	NO
	AG3H93	no	no	no	no	can't be computed:>50% are one size class
	AGXH678	no	no	no	no	NO
	AG3H119	no	no	no	no	NO
	AG3H555	no	no	no	no	NO
	AG3H249	no	no	no	no	NO
	XND5D1	no	no	no	no	NO
	AG3H59	no	no	may be present ;excess homozygous	yes	NO
	AG3H577	no	no	no	no	NO
	AG3H758	no	no	no	no	NO
	XND5C1	no	no	may be present ;excess homozygous	yes	can't be computed:>50% are one size class
	XND6U2	no	no	no	no	can't be computed:>50% are

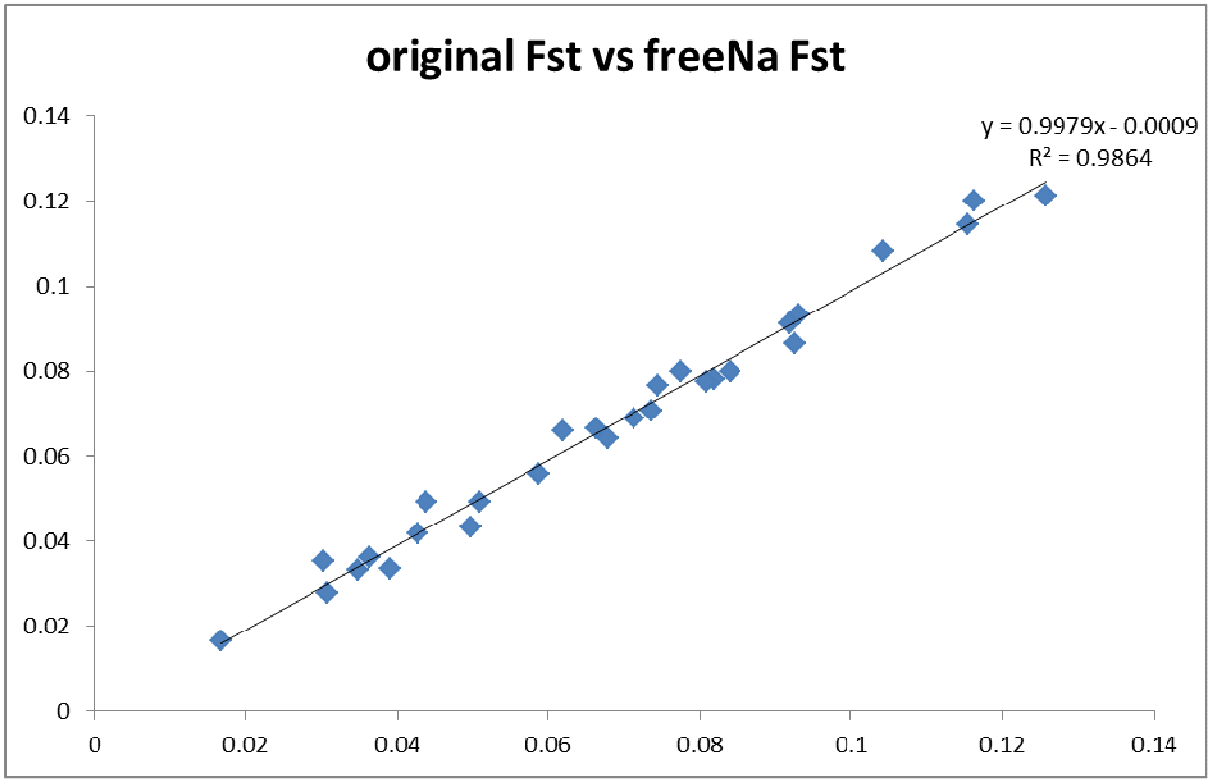
						one size class
SenWass	AG3H242	no	no	no	no	can't be computed:>50% are one size class
	AG3H128	no	no	no	no	NO
	AG3H93	no	no	may be present ;excess homozygous	yes	YES
	AGXH678	no	no	may be present ;excess homozygous	yes	NO
	AG3H119	no	no	may be present ;excess homozygous	yes	YES
	AG3H555	no	no	may be present ;excess homozygous	yes	YES
	AG3H249	no	no	no	no	NO
	XND5D1	no	no	no	no	NO
	AG3H59	no	no	no	no	NO
	AG3H577	no	no	no	no	can't be computed:>50% are

					one size class
AG3H758	no	no	may be present ;excess homozygous	yes	YES
XND5C1	no	no	no	no	NO
XND6U2	no	no	no	no	can't be computed:>50% are one size class

Appendix 24 Average cluster membership for cluster analysis for each K=2 and K=3, run in STRUCTURE

	inferred clusters					
	k=2			k=3		
Sample site	1	2	N	1	2	3
AB	0.207287	0.792713	94	0.735564	0.069575	0.194777
CD	0.334248	0.665752	109	0.227147	0.444532	0.328266
EF	0.988796	0.011204	49	0.00851	0.012388	0.979082
JK	0.987786	0.012214	28	0.004429	0.017393	0.978214
LM	0.668633	0.331367	120	0.014433	0.326392	0.659058
SenBadi	0.994917	0.005083	12	0.005667	0.00375	0.990583
SenBarkeyel	0.993556	0.006444	9	0.006444	0.005222	0.988222
SenWassadou	0.992233	0.007767	30	0.008633	0.004533	0.986533

Appendix 25 Scatter plot of pairwise F_{ST} estimated from original data and null-corrected data in FreeNA



Appendix 26 First generation immigrants as identified by GENECLASS and GenAlex using Paetkau algorithm

Pop	Membership	Year	Mutant	Wild	N
AB	migrant	2010	0	0	0
		2013	0	2	2
		2014	0	19	19
	resident	2010	0	0	0
		2013	14	53	67
		2014	0	6	6
CD	migrant	2010	0	0	8
		2013	1	28	0
		2014	3	14	0
	resident	2010	0	0	4
		2013	0	13	0
		2014	16	34	0
EF	migrant	2010	0	0	4
		2013	13	4	0
		2014	1	0	0
	resident	2010	0	0	5
		2013	21	6	0
		2014	2	2	0
JK	migrant	2010	0	0	0
		2013	4	0	29
		2014	0	0	17
	resident	2010	0	0	0
		2013	24	0	13
		2014	0	0	50
LM	migrant	2010	0	0	0
		2013	14	1	17
		2014	42	4	1
	resident	2010	0	0	0
		2013	43	1	27
		2014	15	0	4
BAD	migrant	2010	6	2	0
		2013	0	0	4
		2014	0	0	0
	resident	2010	3	1	0
		2013	0	0	24
		2014	0	0	0
BAR	migrant	2010	4	0	0
		2013	0	0	15
		2014	0	0	46
	resident	2010	0	5	0

		2013	0	0	44
		2014	0	0	15
WAS	migrant	2010	2	1	3
		2013	0	0	0
		2014	0	0	0
	resident	2010	22	1	23
		2013	0	0	0
		2014	0	0	0